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Toxicity of mixtures of cocaine and often co-consumed substances

Dissertation thesis for the Master Degree in Analytical Clinical and Forensic Toxicology

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ABSTRACT

Cocaine (COC) is frequently misused with other substances; among them, the most prominent are ethanol (EtOH), heroin (HER) or its metabolite morphine (MOR), and 3,4-methylenedioxymethamphetamine (ecstasy or MDMA). Cardiac and hepatic toxicity are ominous complications of these drugs in humans, and may be further aggravated by their combination. So, in the current work we sought to assess the hepatotoxic and cardiotoxic mixture effects of COC combined with EtOH, HER/ MOR, or MDMA.

For this purpose, COC was combined with HER/MOR (Mix A/ Mix B), EtOH (Mix C) or MDMA (Mix D) at the same drug ratio found in the blood of intoxicated abusers or in seized ecstasy pills. Then, primary cultured hepatocytes isolated from Wistar Han rats and H9c2 rat cardiomyocytes were exposed to the individual drugs or their combinations. After 24h, cytotoxicity was recorded and mixture expectations predicted by concentration addition (CA) and independent action (IA) models. Changes in the intracellular contents of reduced (GSH) and oxidised (GSSG) glutathione, adenosine triphosphate (ATP), reactive species of oxygen and nitrogen (ROS/RNS), and in the mitochondrial membrane potential ($\Delta\Psi m$) were also evaluated.

Primary hepatocytes were more susceptible than H9c2 cardiomyocytes to the drug-induced toxicity. HER (EC_{50} 0.26 mM) and MDMA (EC_{50} 1.07 mM) revealed to be the most toxic drugs, in primary hepatocytes and H9c2 cells, respectively. The toxicity predicted by CA and IA was roughly coincident; except for Mix C cardiotoxicity, none of the models predicted mixture effects accurately. This was probably a consequence of alterations on pharmacokinetics/ dynamics pathways, when drugs were in association. Increased ROS/RNS and GSSG, depletion of GSH and energetic stores, and mitochondrial hyperpolarization seemed to be involved in the observed cardiotoxicity.

Overall, the presence of other drugs greatly altered COC individual toxicity as several synergisms and additive effects were observed. Both occurrences are worrisome and, in a clinical perspective, may significantly deteriorate the health of abusers. Of concern, the toxicological impact of the co-occurrence of COC and other substances could be hardly anticipated by the most broadly employed models for the calculation of mixture effects of chemicals. Evidence from our work also indicates that the effects of a particular mixture might not be consistent among distinct organs, suggesting that, depending on the functional specificity of the target sites, differential mechanisms of toxicity/ protection might be activated.

Keywords: Cocaine; Heroin/ Morphine; 3,4-Methylenedioxymethamphetamine (*ecstasy*, MDMA); Ethanol; *In vitro* toxicity.

RESUMO

A cocaína (COC) é frequentemente misturada com outras substâncias, entre estas, as mais utilizadas são o etanol (EtOH), a heroína (HER) ou o seu metabolito morfina (MOR) e a 3,4-metilenodioximetanfetamina (ecstasy ou MDMA). A hepatotoxicidade e a cardiotoxicidade são das complicações mais proeminentes destas drogas em humanos, as quais podem ser exacerbadas pela combinação destas substâncias. Assim, no presente trabalho pretendeu-se avaliar os efeitos hepatotóxicos e cardiotóxicos da mistura da COC com o EtOH, a HER/ MOR, ou a MDMA.

Para este efeito, a COC foi combinada com a HER/MOR (Mix A/ Mix B), o EtOH (Mix C) ou a MDMA (Mix D) na proporção encontrada no sangue de indivíduos intoxicados ou em pastilhas de ecstasy apreendidas. De seguida, hepatócitos primários isolados de ratos Wistar Han e cardiomiócitos de rato H9c2, foram expostos às substâncias individualmente e em combinação. Após 24h, a citotoxicidade foi avaliada e os efeitos de mistura previstos pelos modelos de adição concentração (CA) e ação independente (IA). Alterações a nível do conteúdo intracelular da glutationa reduzida (GSH) e oxidada (GSSG), da adenosina trifosfato (ATP), das espécies reativas de oxigénio e nitrogénio (ROS/RNS) e do potencial da membrana mitocondrial ($\Delta\Psi m$) foram também avaliadas.

Os hepatócitos primários demonstraram ser mais suscetíveis do que o cardiomiócitos H9c2 à toxicidade induzida por estas substâncias. A HER (EC_{50} 0,26 mM) e o MDMA (EC_{50} 1,07 mM) revelaram ser as substâncias mais tóxicas, nos hepatócitos primários e nas células H9c2, respetivamente. Para todas as misturas testadas, a toxicidade prevista por AC e AI foi praticamente coincidente, à exceção da Mix D, quando testada em hepatócitos primários. No entanto, nenhum dos modelos conseguiu prever com precisão os efeitos de mistura observados, provavelmente, uma consequência de alterações nas vias farmacocinéticas/ dinâmicas destas substâncias, quando concomitantemente consumidas. O aumento de ROS/RNS e GSSG, a diminuição de GSH e das reservas energéticas, e hiperpolarização mitocondrial parecem estar envolvidos na cardiotoxicidade observada.

Em suma, os nossos resultados indicam que a presença de outras substâncias podem alterar significativamente a toxicidade individual da COC, uma vez que foram observados vários sinergismos e efeitos aditivos. Estes efeitos são preocupantes e podem deteriorar significativamente a saúde dos consumidores. Igualmente preocupante é a dificuldade em antecipar o impacto toxicológico da coadministração da COC e outras substâncias por modelos que são amplamente utilizados para o cálculo dos efeitos de misturas. Adicionalmente, o nosso trabalho evidencia que os efeitos de uma mistura podem não ser consistentes nos diferentes órgãos, o que sugere que a toxicidade pode depender

da especificidade funcional dos locais alvo das substâncias e, consequentemente, da ativação diferencial de mecanismos (des)toxificantes.

Palavras-chave: Cocaína; Heroína/ Morfina; 3,4-Metilenedioximetanfetamina (ecstasy, MDMA); Etanol; Toxicidade *in vitro*.

INDEX

INDEX OF TABLES

INDEX OF FIGURES

ABBREVIATIONS

5-HT – Serotonin

ATP – Adenosine-5'-triphosphate

BBB – Blood brain barrier

BE – Benzoylecgonine

BP - Blood pressure

BSA – Bovine serum albumin

CA – Concentration addition

$CaCl_2$ – Calcium dichloride

CE – Cocaethylene

CNS – Central nervous system

COC – Cocaine

$CuSO_4$ – Cupper sulphate

CYP450 – Cytochrome P450

DA – Dopamine

DAT – Dopamine transporter

DCFH – 2',7'-Dichlorofluorescein

DCFH-DA – 2-7-Dichlorofluorescin diacetate

DTNB – 5,5'-Dithiobis(2-nitrobenzoic acid)

ED – Ecgonidine

EDEE – Ecgonidine ethyl ester

EDME – Ecgonidine methyl ester

EDTA – Ethylenediamine tetraacetic acid

EEE – Ecgonine ethyl ester

EGTA – Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EMCDDA – European Monitoring Centre for Drugs and Drug Addiction

EME – Ecgonine methyl ester

ERK – Extracellular signal-regulated kinase

EtOH – Ethanol

FBS – Fetal bovine serum

GABA – γ-Aminobutyric acid

GIRK – Potassium channels activated by G protein

GPX – Glutathione peroxidase

GR – Glutathione reductase

GSH – Reduced glutathione

GSHt – Total glutathione

GSSG – Oxidized glutathione

GST – Glutathione-S-transferase

H_2O_2 – Hydrogen peroxide

HBSS – Hank's balanced salt solution

hCE1 – Human carboxylesterase type 1

hCE2 – Human carboxylesterase type 2

$HClO_4$ – Perchloric acid

HER – Heroin

HIV – Human immunodeficiency virus

HPTECs – Human proximal tubular epithelial cells

HR – Heart rate

i.n. – Intranasal

i.v. – Intravenous

IA – Independent action

KH_2PO_4 – Potassium dihydrogen phosphate

$KHCO_3$ – Potassium bicarbonate

$KNaC_4H_4O_6.4H_2O$ – Potassium sodium tartrate tetrahydrate

LDH – Lactate dehydrogenase

MAPK – Mitogen-activated protein kinase

$MgSO_4$ – Magnesium sulphate

MnSOD – Manganese superoxide dismutase

MOR – Morphine

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NA – Noradrenaline

Na_2CO_3 – Sodium carbonate

Na_2HPO_4 – Disodium phosphate

NaCl – Sodium chloride

NADH – Nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate

$NaHCO_3$ – Sodium bicarbonate

$NaHPO_4$ – Sodium phosphate monobasic

NaOH – Sodium hydroxide

NAT – Noradrenaline transporter

NBE – Norbenzoylecgonine

NCE – Norcocaethylene

NCOC – Norcocaine

NCOC-NO. – Norcocaine nitroxide

NEAA – Non-essential amino acids

NEDME – Norecgonidine methyl ester

NEME – Norecgonine methyl ester

NMDA – N-methyl-D-aspartate

N-OH-NCOC – N-Hydroxynorcocaine

OH-BE – Hydroxybenzoylecgonine

OH-COC – Hydroxycocaine

PChE – Pseudocholinesterase

ROS – Reactive oxygen species

SERT – Serotonin transporter

TMRE – Tetramethylrhodamine ethyl ester perchlorate

TNB – 5-Thio-2-nitrobenzoic acid

TNF-R1 – TNF-α Receptor-1

TNF-α – Tumour necrosis factor-alpha

USA – United States of America

VD – Volume of distribution

WBCs – White blood cells

β-NADH – β-Nicotinamide adenine

PART I

INTRODUCTION

1. Introduction

1.1. Cocaine (COC): Historical perspective

Cocaine (COC) or benzoylmethylecognine is a coca alkaloid extracted from the leaves of Erythroxylon coca plant , which is found in the high mountain ranges of Bolivia, Colombia, and Peru (Freye & Levy, 2009). Although the modern incarnation of COC only occurred in the 1860s, coca plant had been cultivated as far back as 3000 BC by indigenous people from South America, who used to chew the leaves of the plant in magical ceremonies and initiation rites due to its psychoactive and stimulating properties (Freye & Levy, 2009; Volkow, 2009). and ancient Incas used its leaves.

In 1859, COC was synthesized for the first time by the German chemist Albert Nieman. In 1863, Angelo Mariani, a Corsican chemist, created Vin Mariani, a wine product containing coca leaves extract (unpurified COC). This new tonic achieved success in Europe and became a drink of choice to famous personalities, such as writers, compositors, and royal members (Freye & Levy, 2009; Karch, 1999). In 1884, the Austrian psychoanalysis founder Sigmund Freud published an article about the benefits of the drug; he was the first defending the healing effects of COC, in cases of depression (Freye & Levy, 2009; Volkow, 2009). In the late nineteenth century, coca extract products were considered a boon to medicine; they were used as a muscle relaxant, and as remedies to treat respiratory ailments, such as asthma and whooping cough, in which other medical treatments wee ineffective. However, with the advantage came the problem and many users became addicted; for example, some people who used COC to combat morphine (MOR) dependence became addicted to both (Freye & Levy, 2009).

Later, in 1886, Atlanta introduced legislation against the consumption of alcoholic beverages and a non-alcoholic version of his popular beverage was developed by introducing a syrup containing extracts of fruits from the kola and coca leaves, which is currently known as Coca-Cola. This soft drink is the most popular in history as it produces euphoric and energizing effects on consumer (Freye & Levy, 2009; Volkow, 2009). Although the soft drink has kept the same name, nowadays Coca-Cola does not contain COC. In 1904, when the dangers of the drug became more evident, public pressure forced Coca-Cola Company to remove COC from the beverage. However, there is some evidences that decocanized coca leaf extracts are still used.

The early twentieth century was marked by an increase in COC consumption, particularly among labourers, the youth, and the urban underworld; and also by an increase in the perils associated. In the year of 1912, 5,000 COC-related deaths occurred in the

United States of America (USA), fomenting calls for prohibition. In 1920, the drug was officially banned (Freye & Levy, 2009).

In the current century, COC had become the second most trafficked illegal drug in the world, after cannabis (EMCDDA, 2012).

1.2. Cocaine (COC) trading: presentation forms, routes of administration and prevalence of consumption

COC is consumed as two different chemical forms, the water-soluble hydrochloride salt and the water-insoluble COC free base. The choice of one form to the detriment of the other one depends on the social settings of the users.

The hydrochloride salt, the powder form of COC, is typically snorted or injected. On the street, it is known as "*coke*", "*C*", "*snow*", "*flake*" or "*blow*" and can be diluted with inert substances, such as talcum powder or sugar, or with active substances, such as procaine, amphetamine, lidocaine, and opioids (Volkow, 2009). This form of COC is the one clinically used to promote anaesthesia.

The COC freebase is the smoked form of the drug. The hydrochloride salt is processed with ammonia or sodium bicarbonate ($NaHCO_3$) and water, and then heated to remove hydrochloride (EMCDDA, 2007). On the street, COC freebase is denominated as '*crack*', due to the crackling sound produced when the mixture is smoked (Volkow, 2009).

There is a critical heterogeneity among COC users. It is believed that a high number of people use COC sporadically (Van der Poel, Rodenburg, Dijkstra, Stoele, & Van de Mheen, 2009). Only a low number of abusers are addicted users, who inject COC or smoke *crack*. They represent an urban phenomenon: most are homeless, unemployed, sex workers, and heroin (HER) addicts (EMCDDA, 2002). Contrary to these COC heavy users, who are socially marginalized or belong to disadvantage groups, there are also some regular users that are individuals integrated in society, who consume COC only at weekends, parties or other special occasions (EMCDDA, 2012). These users are mainly males, who snort powder COC and use the drug alone or in combination with cannabis or ethanol (EtOH).

About 15.5 million Europeans have used COC at least once in a lifetime; 4.6% of the adults (aged 15-64 years), and 6.3% of the young adults (aged 15-34) (EMCDDA, 2012). The European countries with higher prevalence of COC abuse among the youth are Spain (4.4%) and the United Kingdom (4.2%). Not surprisingly, these two countries present consumption levels similar to Australia (4.8%) and the USA (4.0% among 16-34-year-old adults) (EMCDDA, 2012). In fact, along with Netherlands, Portugal, and Belgium, these are

the principal points of entry for COC into Europe, where in 2005 were seized 107 tons of the drug – half of this amount was apprehended in Spain (EMCDDA, 2007, 2012).

1.3. Pharmacological and toxicological mechanisms of action

1.3.1. Peripheral nervous system

COC acts peripherally as a local anaesthetic mainly by inhibiting sodium influx through specific ion channels in the neuronal cell membrane, in particular the so-called voltage-gated sodium channels (Goldstein, DesLauriers, & Burda, 2009).

Following nerve stimulus, the sodium channels present at the neuronal membrane open, allowing sodium ions to migrate into the cell, causing depolarization. The change on the voltage difference created between the inside and the outside of the axon needs to be counteracted, so potassium ions move out of the axon to repolarize the membrane and the impulse travels down the axon to the terminal, where it signals other neurons. COC has great affinity to the receptors located at the cytoplasmic portion of the sodium channel, and is highly effective in preventing them from opening. When the influx of sodium is interrupted, an action potential cannot arise and the transmission of nerve impulses is blocked (Brown, Prager, Lee, & Ramsey, 1992; Freye & Levy, 2009; Goldstein *et al*., 2009; Hollander, 2008). COC active metabolites, such as norcocaine (NCOC), are also capable of similar interaction with the sodium channels (Matthews & Collins, 1983).

Of note, COC is the only local anaesthetic that exhibits vasoconstriction properties. All other synthetic local anaesthetics are usually mixed with a vasoconstrictor to extend the duration of anaesthesia (Freye & Levy, 2009).

1.3.2. Central nervous system

The central nervous system (CNS) is a main COC target (Goldstein *et al*., 2009). Contrarily to other local anaesthetics that elicit CNS depression at low doses and CNS stimulation at high doses, COC provokes euphoria and alertness at low doses and aggressiveness, disorientation, and hallucinations at high doses (Goldstein *et al*., 2009).

COC pharmacological and toxicological effects at the CNS are a consequence of the high affinity of the drug to the receptors of serotonin (5-HT) and of the catecholamines noradrenaline (NA) and dopamine (DA) (Carroll *et al.*, 1992; Freye & Levy, 2009; Heard, Palmer, & Zahniser, 2008). COC impairs the adrenergic and dopaminergic systems by binding the respective receptors and blocking the reuptake of 5-HT, NA, and DA, this way

increasing their concentration in the synaptic cleft. For instance, in the dopaminergic neuron, COC binds to DA transporter (DAT) on the membrane of pre-synaptic neurons, blocking the removal of DA from the synaptic cleft and its further degradation by monoamine oxidase (MAO) in the nerve terminal (Caine *et al*., 2002; Dackis & O'Brien, 2001; Freye & Levy, 2009; Schilstrom *et al.*, 2006). The DA is then free to bind to its receptors on the post synaptic membrane, producing further activation of dopaminergic neurons.

The addiction and reward effects of COC might be related with the activation of serotonergic pathways, but they are mostly a result of excessive dopaminergic activity on the ventral tegmental area, the *nucleus accumbens* and the caudate nucleus (Freye & Levy, 2009; Goldstein *et al.*, 2009; Sora *et al*., 2001). The increased dopaminergic activation of these areas, known as the reward pathway of the brain, leads to the feeling of euphoria and the 'high' associated with use of the drug. Chronic COC abusers have a lower number of DA receptors, compared with age- and gender-matched controls. They also have decreased dopaminergic activity, which results in impairment of the hedonic function (Dackis & Gold, 1985; Dackis & O'Brien, 2001; Volkow, Fowler, & Wang, 2003; Volkow, Fowler, Wang, & Swanson, 2004). Drug abusers have, therefore, difficulty to be stimulated by non-drug-related environmental stimuli (Volkow *et al.*, 2004).

Although COC abusers present a higher amount of DAT, the receptor levels normalize after interrupting consumption (Malison *et al*., 1998; Volkow *et al.*, 2004).

1.3.3. Cardiovascular system

COC induce several cardiovascular complications, including myocardial ischemia or infarction, coronary vasospasm, accelerated coronary atherosclerosis, coronary thrombosis, and hypertension (Afonso, Mohammad, & Thatai, 2007; Aslibekyan, Levitan, & Mittleman, 2008; Awtry & Philippides, 2010; Hollander, 2008; Kloner, Hale, Alker, & Rezkalla, 1992; Restrepo *et al.*, 2009; Satran *et al.*, 2005). The mechanisms underlying these effects are mainly provoked either by the blockade of sodium channels, or by the blockade of reuptake of catecholamines in the presynaptic neurons, resulting in a potentiation of the sympathetic effects.

The sympathomimetic activity of COC at the presynaptic adrenergic terminals leads to overstimulation of the alpha-adrenergic receptors in the coronary and peripheral arteries. This effect has several consequences, including vasoconstriction, increased coronary resistance, and decreased coronary blood flow, elevated blood pressure (BP), and increased myocardial wall stress.

COC also blocks the voltage-gated sodium channels in the myocardium, altering the generation and conduction of the action potential and, consequently, extending the PR, QT

and QRS intervals. Despite concomitant sympathomimetic stimulation, these occurrences are responsible for the COC negative inotropic effect (Afonso *et al*., 2007; Goldstein *et al.*, 2009; Kloner *et al*., 1992; O'Leary & Hancox, 2010; Restrepo *et al.*, 2009). Additionally, elevated intracellular calcium concentration in the myocyte are also responsible for the development of cardiac dysrhythmias (Bauman, Grawe, Winecoff, & Hariman, 1994; Hollander, 2008; Restrepo *et al*., 2009).

1.3.4. Liver

COC is bioactivated through metabolism into reactive prooxidant metabolites, which are highly deleterious to the structural and functional integrity of the cell (for more details, see the section '1.4.2 Metabolism'). Due to its relevant role on the metabolism of xenobiotics, the liver is therefore a target organ for the toxicity of the drug (Goldstein *et al*., 2009; Ndikum-Moffor, Schoeb, & Roberts, 1998). Accordingly, the hepatotoxicity promoted by COC is dose- and time-dependent, and directly correlated with cytochrome P450 (CYP 450) activity and the levels of NCOC (Smith, Freeman, & Harbison, 1981).

The hepatic effects of the drug were first observed by Ehrlich *et al*. (1890), in the mice liver. Later, Marks and Chapple (1967) reported the drug hepatotoxicity also in humans by observing abnormal liver test results in HER and COC addicts. In 1987, Perino and collaborators linked these clinical findings to liver necrosis (Perino, Warren, & Levine, 1987). After that, several studies were made and results demonstrate significant necrosis in centrolobular hepatocytes, where the levels of CYP 450 are higher (Kanel, Cassidy, Shuster, & Reynolds, 1990), suggesting a role for metabolism in the toxicity of COC.

1.4. Toxicokinetics

As aforementioned, COC might be consumed as a freebase or as a salt. This aspect will significantly impact the pharmacokinetic properties of the drug (Freye & Levy, 2009; Goldstein *et al*., 2009).

1.4.1. Absorption and distribution

The administration route plays a major role in the bioavailability, intensity and duration of the elicited effects of the drug (Hatsukami & Fischman, 1996). While COC hydrochloride is snorted or intravenously injected, the freebase form (crack) is smoked.

COC bioavailability is total when the drug is intravenously administered. The other administration routes (nasal inhalation and smoking) allow the drug to be rapidly absorbed, with a bioavailability of approximately 90% for smoked COC. When the drug is snorted, the bioavailability is dose-dependent and ranges from 25 to 94% (Freye & Levy, 2009; Goldstein *et al*., 2009; Hatsukami & Fischman, 1996; Heard *et al*., 2008). The bioavailability of COC per os is about 30% (Goldstein *et al*., 2009; Hatsukami & Fischman, 1996).

COC effects are sensed 7-10 seconds after smoking, as the drug is quickly absorbed through the extensive network of pulmonary capillaries. Therefore, crack has great popularity due to the rapid onset of its euphoric effects. Similarly, COC effects are almost instantaneously sensed after i.v. administration (30-45 seconds after administration) (Glauser & Queen, 2007; Tashkin, Kleerup, Koyal, Marques, & Goldman, 1996). The intranasal (i.n.) administration takes roughly 3-5 minutes to exert effect, while oral ingestion produces effects only 15 minutes after intake (absorption is slower) (Freye & Levy, 2009).

The smoked form of COC is very dangerous because the time needed to peak subjective effects is very short. Also, the peak plasma concentration is reached within few minutes of the dose administration (approximately 5 minutes later) (Hatsukami & Fischman, 1996). The absorption kinetic of COC is similar for i.v. injected COC hydrochloride and smoked crack COC (Hatsukami & Fischman, 1996).

After absorption, the distribution is rapid and the drug spreads to several organs. COC reaches the highest concentrations in brain, spleen, kidney and lung, followed by heart, blood and muscle (Bortolotti, Gottardo, Pascali, & Tagliaro, 2012). In humans, the volume of distribution fluctuates from 1.96 to 2.7 L/Kg (Goldstein *et al*., 2009; Jeffcoat, Perez-Reyes, Hill, Sadler, & Cook, 1989). After the administration of an i.n. dose of 20 mg, COC could be detected in the serum during 4-6 h; after a 100 mg administration, the drug remained in the serum at least for 12 h (Maurer, Sauer, & Theobald, 2006).

There is evidence that the bioavailability of COC is impacted by gender and, therefore, several studies were made to scrutinize the effects of sex hormones and of menstrual cycle on COC serum concentrations (Lukas *et al*., 1996). Studies demonstrated that male rats present peak COC concentrations around 50% higher than those found in females following i.v. administrations (Festa *et al.*, 2004; Heard *et al*., 2008; Kosten *et al*., 1996).

1.4.2. Metabolism

COC is rapidly metabolized by multiple enzymatic pathways (Figure 1).). The major metabolites are benzoylecgonine (BE) and ecgonine methyl ester (EME), both metabolized to ecgonine (Bortolotti *et al*., 2012; Goldstein *et al*., 2009; Valente, Carvalho, Bastos, de Pinho, & Carvalho, 2012). The metabolite BE results from the enzymatic hydrolysis by human carboxylesterase type 1 (hCE1) in human liver, and represents nearly half of the absorbed dose; while EME may be formed when COC is metabolized by human carboxylesterase type 2 (hCE2) or by pseudocholinesterase (PChE). As EME has low pharmacological effects, individuals presenting low PChE activity sense the effects of COC for longer periods (Bortolotti *et al*., 2012; Goldstein *et al.*, 2009; Valente *et al*., 2012). N-demethylation of COC by CYP450 produces NCOC, a minor metabolite. This metabolite represents 5% of the absorbed drug and crosses the blood-brain barrier (BBB) to produce clinical effects identical to those of COC. The metabolites EME and BE are unable to cross the BBB and, consequently, of exerting effects at the CNS (Goldstein *et al.*, 2009).

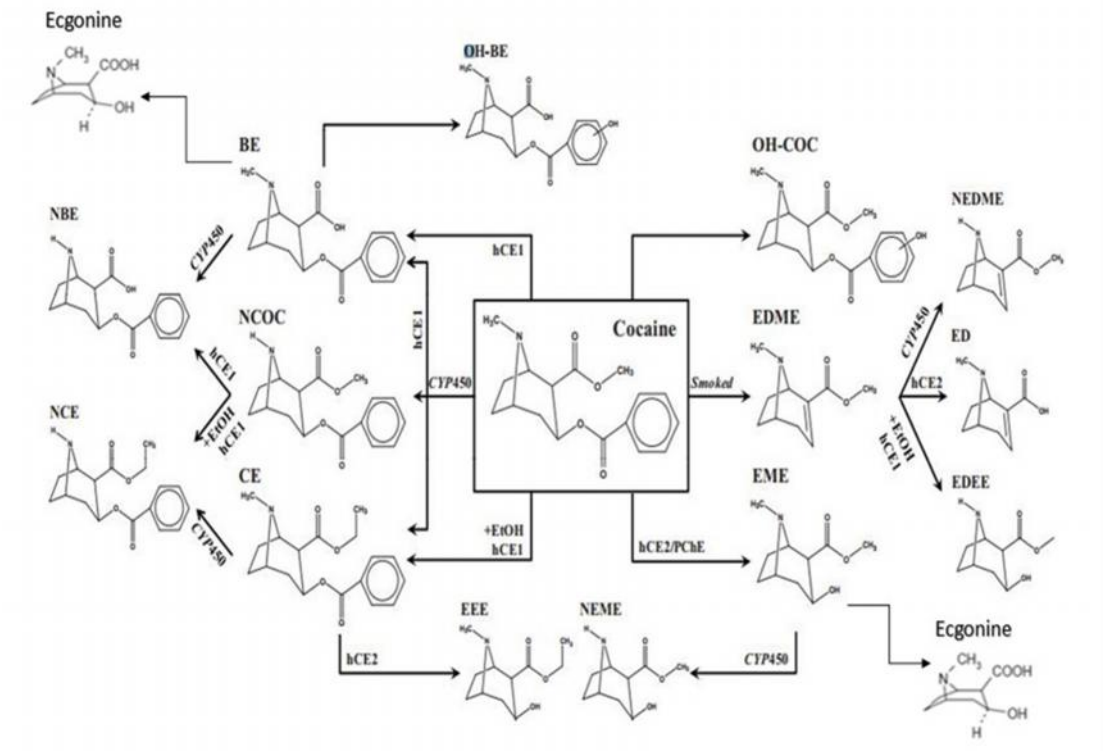

Figure 1. Cocaine metabolism. BE – benzoylecgonine; CE – cocaethylene; ED – ecgonidine; EDEE – ecgonidine ethyl ester; EDME – ecgonidine methyl ester; EEE – ecgonine ethyl ester; EME – ecgonine methyl ester; hCE1 – human carboxylesterase type 1; hCE2 – human carboxylesterase type 2; NBE – norbenzoylecgonine; NCE – norcocaethylene; NCOC – norcocaine; NEDME – norecgonidine methyl ester;

NEME – norecgonine methyl ester; OH-BE – hydroxybenzoylecgonine; OH-COC – hydroxycocaine; PChE – pseudocholinesterase. Adapted from Valente et al. (2012).

The oxidation of NCOC by CYP450 originates N-OH-NCOC, which by turn is metabolized to the free radical NCOC-NO., in the presence of a nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (Pellinen *et al*., 2000). Studies in vitro demonstrated the involvement of NCOC metabolism in COC hepatotoxicity. Two different mechanisms may be at a play. On the one hand, it has been suggested that NCOC-NO. enters in a redox cycle, as it can be reduced to N-OH-NCOC by flavoproteins and, consequently, oxidized to NCOC-NO. by CYP450. This redox cycle can be responsible for the production of reactive species of oxygen (ROS), causing hepatotoxicity via mechanisms of oxidative stress, involving lipid peroxidation (Boelsterli & Goldlin, 1991; Valente *et al.*, 2012). On the other hand, COC-induced hepatotoxicity may involve oxidation of NCOC-NO. to NCOC nitrosonium, a highly reactive compound that binds to hepatic proteins, leading to impairment of the hepatocellular function (Charkoudian & Shuster, 1985). A study made by Ndikum-Moffor *et al.* (1998) presented results consistent with these two mechanisms. By using P450 inhibitors, the authors proved the importance of CYP450 on COC toxicity, as it was responsible for oxidizing N-OH-NCOC to NCOC-NO. and for oxidizing the nitroxide to other reactive species.

Anhydroecgonine methyl ester (AEME) is a metabolite formed when COC is smoked (Figure 1). It can be detected and quantified in different biological fluids, such as urine, sweat and hair; and produces muscarinic effects on the cardiovascular system (Goldstein *et al*., 2009; Kintz, Sengler, Cirimele, & Mangin, 1997). This pyrolysis end product is transformed in ecgonidine (ED) via esterase activity (Bortolotti *et al*., 2012; Caplan, 2015). Along with AEME, ED is used as a biomarker of crack smoking (Cone, Hillsgrove, & Darwin, 1994).

The co-administration of COC and EtOH results in ethylbenzoylecgonine or cocaethylene (CE) (Figure 1), a product formed by hCE1-mediated transesterification of these two drugs in liver (Bortolotti *et al.*, 2012; Goldstein *et al*., 2009). A study demonstrated that 17% of i.v. COC was converted to CE, an active metabolite with a longer half-life (t1/2) than COC (1.7 h and 0.7 h, respectively). Additionally, compared to COC, CE presents higher euphorigenic and reinforcing properties (Bortolotti *et al.*, 2012; Goldstein *et al.*, 2009). This metabolite is an important biomarker of COC and EtOH co-administration and it can be quantified in urine (Politi, Zucchella, Morini, Stramesi, & Polettini, 2007).

The co-administration of crack and EtOH leads to the formation of anhydroecgonine ethylester (AEEE), which can be used as a biomarker of the co-exposure to these two drugs (Myers, Williams, Kraner, & Callery, 2005).

1.4.3. Excretion

COC is mainly excreted in urine, after metabolism; a minor percentage of the drug is excreted in faeces. Approximately 9.5-20% of unchanged COC is eliminated in urine and may be detected 24-36 h after consumption. The major metabolites eliminated in urine are EME (32-49%) and BE (35-54%) and can be detected up to 24 h. Along with ecgonine, these metabolites represent 80-90% of urinary metabolites in humans (Bortolotti *et al*., 2012; Goldstein *et al*., 2009).

Research conducted to unveil the disposition and elimination pathways of COC after repeated administration demonstrated that the drug and its metabolites have a biphasic excretion profile. Following the drug withdrawal, an initial elimination phase is immediately observed, presenting an elimination pattern comparable to that observed after acute dosing. A second, long-term elimination phase occurs during abstinence, as a result of a protracted release of COC accumulated in the body. Although the concentrations of COC and metabolites detected in this terminal phase are fairly low, the t1/2 of parent compound and metabolites greatly exceed the t1/2 reported for the first phase and those observed in previous studies on acute COC administration, enabling the drug to be detected in saliva and urine for an extended period after long-term, high-dose administrations (Jufer, Wstadik, Walsh, Levine, & Cone, 2000).

Also with the intent of evaluating the pharmacokinetics of chronic COC administration, Jufer *et al*. (2000) performed a study in healthy volunteers who were given ascending oral doses of COC (from an initial intake of 500 mg/day up to 2 g/day). The authors reported mean plasma, saliva and urine COC elimination t1/2 of 1.5 h, 1.2 h and 4.1 h, respectively, for the first elimination phase. These values were mostly similar to those observed for COC elimination t1/2 after acute dosing. In 50% of the tested subjects, the second elimination t1/2 for COC was 19h. It is possible that a biphasic elimination profile was also present for the remaining subjects, but it was difficult to confirm the existence of a second elimination phase due to the interference caused by high concentrations of BE that obfuscated COC quantification. During the terminal elimination phase, the urinary t1/2 of metabolites were estimated to range from 14.6 to 52.4 h (t1/2 substantially longer than previously estimated). This biphasic elimination pattern for COC and its metabolites suggests that the drug accumulates in the body during chronic exposure.

As a lipophilic drug, COC is likely to be deposited in the adipose tissues, where it has already been detected in post-mortem cases of COC poisoning (Jufer *et al.*, 2000; Moolchan, Cone, Wstadik, Huestis, & Preston, 2000). Additionally, detection of COC and its metabolites can be performed on several other biological specimens, such as hair, sweat, meconium, saliva, and amniotic fluid.

1.5. Polydrug abuse

. As a club drug, COC use is linked with simultaneous consumption of other illicit and licit drugs, such HER and EtOH. This polydrug abuse pattern is intended to increase or modify the psychoactive effects of the drug, to leaven or compensate for its negative effects and/or to supplement for it when supply is low (Kelly & Parsons, 2008). According to Pakula *et al.* (2009), the combined use of COC might be instigated by circumstantial and functional reasons (Figure 2).

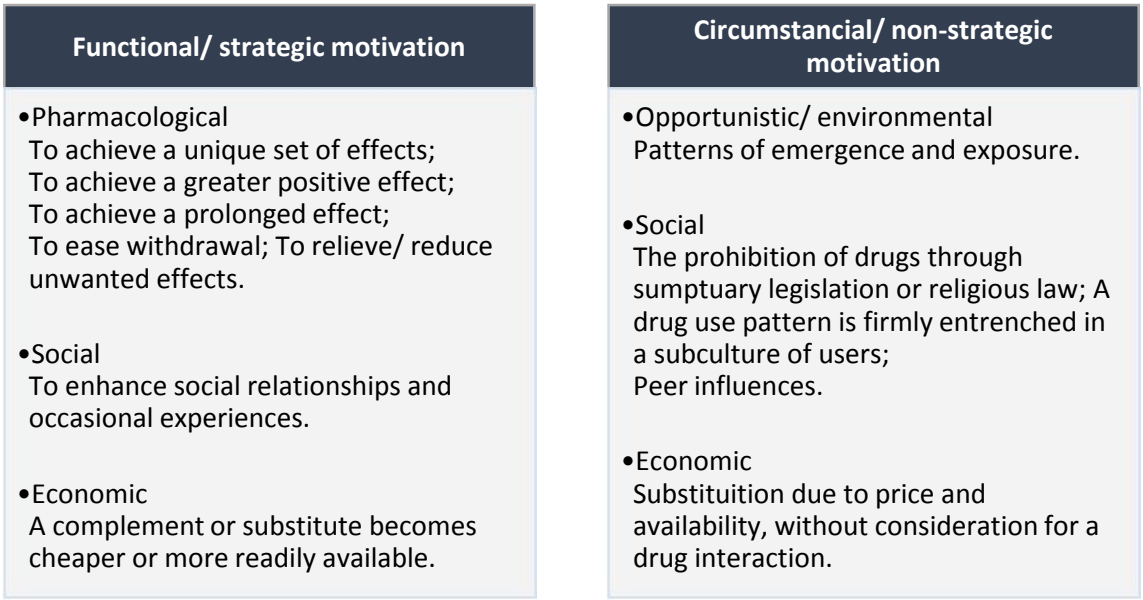

Figure 2. A conceptual model for understanding the reasons underlying polydrug abuse. Adapted from Pakula et al. (2009).

In circumstantial models, the choice of combining different drugs depends on environmental constraints and influences, while in functional models the consumer chooses substances based on aims or expectancies (Pakula, Macdonald, & Stockwell, 2009). Often, the selection of the drug combination is based on descriptions made by other users on the duration and on the subjective effects perceived during pleasurable drug experiences (EMCDDA, 2002). However, individual characteristics or environmental and social factors may severely impact the pharmacological and toxicological effects of the drugs. In addition, as a specific substance may interact with the body in a different and completely

unpredictable way when it is in the presence of other drugs, polydrug abuse is frequently related to health-threatening drug reactions (EMCDDA, 2002; Kelly & Parsons, 2008).

Also, the choice of co-misused psychoactive substances depends on local availability, personal preferences, fashion, and other factors (EMCDDA, 2009). Cannabis and EtOH are the substances most combined with COC. In 2003, the *European School Survey Project on Alcohol and Other Drugs* (ESPAD) demonstrated high-prevalence rates of COC co-use with cannabis, among young students (15 to 16 years old) (EMCDDA, 2009; Olszewski, Matias, Monshouwer, & Kokkevi, 2009). Opiates and tobacco are also frequently co-consumed with the drug (Pakula, Macdonald, & Stockwell, 2009). In some countries, 67-89% of COC users have consumed cannabis concomitantly, while more than 60% have used amphetamines (50% have used *ecstasy*). On the other hand, co-consumption of EtOH is practiced by 14-58% of COC users (Table 1) (EMCDDA, 2009).

Table 1 Prevalence of drugs used among cocaine abusers. Data are presented as the mean of results from 33 European countries. Adapted from EMCDDA (2009).

Co-abused drug	Prevalence of use (%)
Cannabis	65.1
Ecstasy	53.6
Heroin	40.7
Amphetamines	35.6
Hallucinogenic mushrooms	35.3
LSD	35.0

It is possible that polydrug misuse is influenced by gender, as studies demonstrated higher prevalence among young males. On the other hand, no significant relationship was established between polydrug abuse and the mental health condition of the abuser (Table 2) (Kelly & Parsons, 2008).

Table 2. Relationship between polydrug abuse and mental health condition of abusers (n=361). The values represent the odd ratios. Adapted from Kelly et al. (2008)

Mental disorder	Polydrug users	Non-polydrug users
Depression	0.87	0.91
Anxiety	0.78	0.85
Stress	2.70	2.55
Coping ability	3.68	3.65
Problem drinking	12.49	13.64

1.5.1. Cocaine (COC) and ethanol (EtOH) co-use

Some studies indicate that up to 90% of COC abusers also consume EtOH (Czoty, 2015; Helzer & Pryzbeck, 1988; Kampman *et al*., 2013). The use of EtOH with COC is considered dangerous, as the combination can be more cardiotoxic and hepatotoxic than EtOH or COC alone (EMCDDA, 2002). EtOH induces CYP 450 and its chronic use is capable of potentiating COC-induced liver damage (*e.g.*, severe centrolobular hepatic necrosis) (Smith *et al*., 1981). Concomitant use of COC and EtOH increases levels of COC in blood (about 30%) and produces CE, a metabolite with longer $t_{1/2}$ (Bortolotti *et al*., 2012). Pharmacological and physiological effects of CE and COC are similar; these two substances increase dopaminergic activity by activating mesolimbic pathways. In *nucleus accumbens*, it was shown that both COC and CE block DA reuptake, resulting in increased extracellular levels of DA (Czoty, 2015; Sobel & Riley, 1999). As these substances increase BP and heart rate (HR), they can increase EtOH consumption by masking EtOH intoxication effects (EMCDDA, 2009; Farre *et al*., 1997). In humans, administration of COC following EtOH intake decreases BE levels in urine, whereas increases CE levels (Harris, Everhart, Mendelson, & Jones, 2003).

The mechanisms of interaction of CE with COC or with EtOH are yet to be completely disclosed, but some authors claim that in humans the interaction with COC is additive; CE can be transesterified to COC, in the presence of methanol, by hepatic esterases, which results in increased levels of COC, prolonging the action the parent drug (Bailey, 1994). Therefore, the *dose addition* effect reported for CE with COC and for CE with EtOH could help explain the higher physiological and pharmacological effects observed, compared to the consumption of EtOH or COC alone.

A further study conducted in rats aimed to analyse the behavioural interactions of CE with COC and with EtOH on schedule-controlled responding. The authors used drug-

experienced rats that were previously administered CE, COC, and EtOH alone, and measured the rate of drug licking. Then, they combined CE with COC or with EtOH and demonstrated that the dose-response curves for COC and EtOH alone were shifted to the left and down by the presence of CE. Also the CE curve was displaced to left and down by both COC and EtOH (Figure 3 and 4). These results confirmed that CE interacts with EtOH and with COC, increasing the effect of the individual drugs (Sobel & Riley, 1999). The authors argued for the occurrence of an additive interaction underlying the effects observed for the mixture of CE with COC or with EtOH, as the isobolographic analysis revealed that the ED_{50} values for the combinations CE–EtOH and CE–COC did not significantly differ from the additive calculations, suggesting that these two drugs work by a common mechanism (Sobel & Riley, 1999).

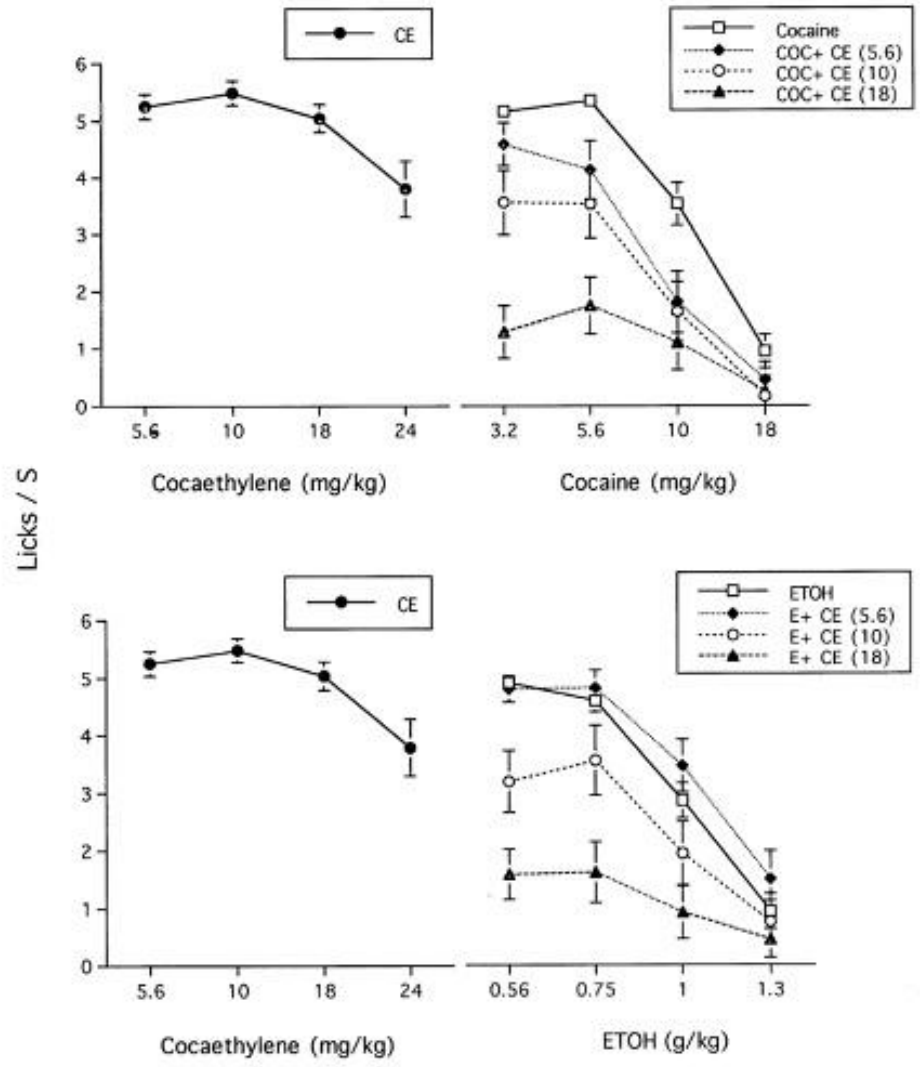

Figure 3. Dose-response relationships for cocaine (top) and ethanol (bottom) administered alone or in combination with cocaethylene. Statistically significant differences ($p<0.05$) were observed between the individual and combined administrations for all doses tested (5.6; 10; 18 mg/kg). Retrieved from Sobel & Riley (1999).

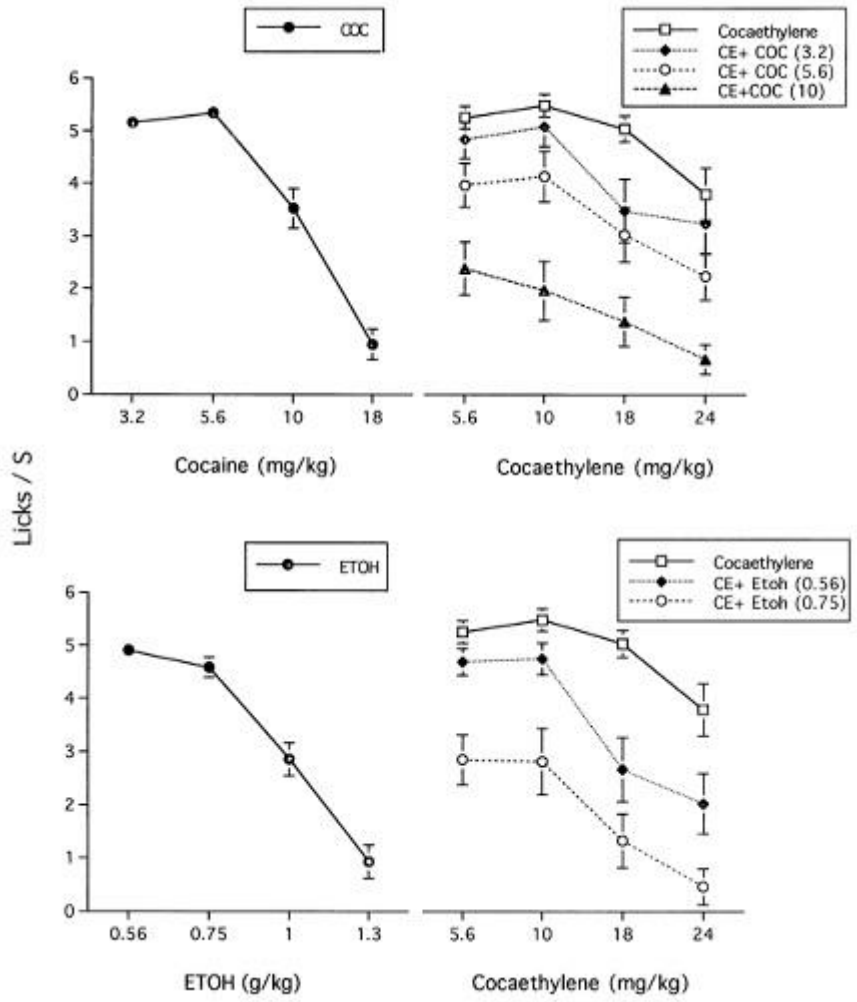

Figure 4 Dose-response relationships for cocaethylene administered alone and in combination with cocaine (top) and ethanol (bottom). Statistically significant differences ($p<0.05$) were observed between the individual and combined administrations of cocaine (5.6 mg/kg and 10 mg/kg) for all doses tested of cocaethylene (5.6; 10; 18 mg/kg). Statistically significant differences ($p<0.05$) between the individual and combined administrations of 0.75 g/kg ethanol were observed for all doses tested of cocaethylene (5.6; 10; 18 mg/kg) and for administrations of 0.56 g/kg ethanol with 18 mg/kg cocaethylene. Retrieved from Sobel & Riley (1999).

The co-administration of COC with EtOH has been involved in several fatalities (Pilgrim, Woodford, & Drummer, 2013) and the cardiovascular toxicity that arise from this combination was implicated in the cause of the majority of these deaths (Garfia, Valverde, Borondo, Candenas, & Lucena, 1990). The co-administration of COC and EtOH is particularly dangerous because these drugs activate the sympathetic nervous system through the increase of catecholamines, consequently increasing HR and BP (Garfia *et al*., 1990). In Table 3 are compiled results from a few studies performed to evaluate the consequences of EtOH intake on COC pharmacokinetics.

In opposition to EtOH administration, which is ingested *per os*, different forms and routes of administering COC are used, as already mentioned (Pakula, Macdonald, & Stockwell, 2009). A study made by Gossop *et al.* (2006) demonstrated differences in the effects attained when smoking *crack* or snorting COC with EtOH. *Crack* users drink less EtOH at the end of *crack*-using sessions, while users who insufflate COC take increased doses of the drug and EtOH. These differences can be related to the different impact of the administration route on the pharmacokinetics of the drug (absorption and bioavailability) and on the balance of euphoric/ dysphoric effects. In general, EtOH is often used when COC is snorted (Pakula, Macdonald, Stockwell, & Sharma, 2009), and these users are more susceptible to heavy drinking and to serious adverse effects than *crack* abusers (Gossop *et al.*, 2006). Snorting COC along with EtOH intake may result in a vicious cycle that ultimately ends up in high consumption of both drugs, as described in a testimony published by Pakula *et al*. *(2009)*:

"If you drink, you get depressed. Then you do line, you get happy and then if you did too much lines and not enough drinking then you'd be off the wall and everyone would know you are screwed up (…). Up down up down up down you can't find a balance which you never find.

Table 3. Studies in volunteers on the co-use of cocaine (COC) and ethanol (EtOH). Blood pressure (BP); cocaethylene (CE); Intanasal (i.n.); Intravenous (i.v.); Hert rate (HR); Volume of distribution (VD).

Number of subjects studied	COC dose (route of administration)	EtOH dose (route of administration)	CE dose (route of administration)	Mean peak values	Mixture effects (compared with COC and EtOH alone)	Reference
9	100 mg (i.n.)	1 g/kg (oral)			Higher COC plasma levels; Increased HR and BP.	(Farre et al., 1993)
12	After 50 minutes of EtOH ingestion was given 1.25 mg/kg (i.n.) or 1.9 mg/kg (i.n.)	0.85 g/kg administered over a 30 minutes period		EtOH blood concentration: 0.092 g/dL; COC plasm concentration: 10.48 (ng/mL); CE plasm concentration: 15.44 (ng/mL).	Increased COC plasm concentration; Increased HR.	(Perez-Reyes & Jeffcoat, 1992)
3	0.25 mg/kg (i.v.)		0.25 mg/kg (i.v.)	-	Tachycardia.	(Perez-Reyes, 1993)
6	0.25 mg/kg (i.v.)		0.25 mg/kg (i.v.)	COC plasm concentration: 170.3±24.1 (ng/mL); CE plasm concentration: 159.6±30.5 (ng/mL).	CE: smaller elimination rate and longer $t_{1/2}$.	(Perez-Reyes, Jeffcoat, Myers, Sihler, & Cook, 1994)
8	0.92 mg/kg (i.n.)		0.48 mg/kg (i.n) or 0.95 mg/kg (i.n)	-	Similar euphoric and cardiovascular effects for COC and CE (HR, BP); CE: smaller VD, slower clearance, longer $t_{1/2}$.	(McCance, Price, Kosten, & Jatlow, 1995)

Number of subjects studied	**COC dose (route of administration)**	**EtOH dose (route of administration)**	**CE dose (route of administration)**	**Mean peak values**	**Mixture effects (compared with COC and EtOH alone)**	**Reference**
8	100 mg/kg (i.n.)	0.8 g/kg (oral)		COC plasm concentration: 330.5±111.7(ng/mL); CE plasm concentration: 48.4±14.7(ng/mL); EtOH plasm concentration: 1003.4±172.4 (ng/mL);	Higher HR and BP.	(Farre et al., 1997)
8	4 doses of 1 mg/kg every 30 min (i.n.)	1 g/kg after the first COC dose; 120 mg/kg at 60 min (oral)		COC plasm concentration: 893±114 (ng/mL); CE plasm concentration: 114±18 (ng/mL); EtOH plasm concentration for first and second administrations: 99±7 mg/dL; 118±10 mg/dL.	Increased cardiovascular effects, including HR and BP, greater euphoria and mental and physical well-being ("*feel good*");	(McCance-Katz, Kosten, & Jatlow, 1998)

1.5.2. Cocaine (COC) and heroin (HER) co-use

COC and opioids can be taken simultaneously or separately, in succession; either by smoking, snorting, or i.v. injection. The simultaneous use of COC and opioids (*e.g.* HER or MOR) is referred to as *speedball* (Rhodes, Briggs, Kimber, Jones, & Holloway, 2007). In general, consumers of *speedball* are socially marginalized users, who smoke or inject COC (Decorte, 2001; Gossop, Griffiths, Powis, & Strang, 1994; Leri, Bruneau, & Stewart, 2003). Co-users that inject these drugs are more prone to risk of being exposed to transmitted infections, such as Human immunodeficiency virus (HIV). They also present higher incidence of personality disorders and worse treatment outcomes (DeMaria, Sterling, & Weinstein, 2000; Downey, Helmus, & Schuster, 2000; Hudgins, McCusker, & Stoddard, 1995; King, Kidorf, Stoller, Carter, & Brooner, 2001). In addition, due to the short $t_{1/2}$ of injectable COC, these health risks are increased by the high frequency of injections (Leri *et al.*, 2003).

HER is more lipophilic than MOR and, consequently, more readily to cross BBB. HER is deacetylated in body by hCE1, hCE2, and PChE, producing active metabolites, such as 6-monoacetylmorphine (6-MAM), 3-monoacetylmorphine (3-MAM) and MOR. The 6-MAM is metabolized into MOR, and is used as biomarker for HER consumption (Andersson, Bjorkhem-Bergman, & Beck, 2015). As HER and COC share the same metabolic pathways (hCE1, hCE2, PChE, and CYP3A4), interactions between these drugs are expected to occur during consumption of *speedball*, affecting the final outcomes attributable to each drug alone (Kamendulis, Brzezinski, Pindel, Bosron, & Dean, 1996).

Recently, chemical interactions between COC and HER (or its metabolites) have been described. Garrido *et al.* (2007) investigated the electrochemical behaviour of mixtures of COC and HER or MOR. The electrochemical study of COC–HER interaction is not available because these drugs have similar potentials (probably because both drugs present only one oxidation peak that represent the anodic oxidation of the tertiary amine group). Results from COC–MOR combination suggest a marked interaction between the drugs, as the oxidation peak of the phenolic group of MOR decreased with increasing concentrations of COC. The authors suggested the formation of a complex or adduct of COC with either MOR or HER, although they acknowledged that COC–HER interaction is more difficult to occur due to the presence of the two terminal $–O(C=O)CH_3$ groups in the molecule of HER that hamper the COC molecule docking; the two aromatic rings of MOR make the molecule more favourable to the interaction with COC because it does not represent any significant steric hindrance. Since the 3-OH group of MOR is structurally unavailable in the adduct form, and it is responsible for the interaction with the opioid

receptors, this interaction probably changes the drug response at this level; note that HER has to be previously deacetylated to promote brain effects.

In rat cortical neurons, sequential and simultaneous exposures to COC and HER presented differences in the activated neurotoxic pathways. Sequential exposures led to a major decrease in metabolic activity and increased cytochrome c release and, consequently, apoptosis through caspase-3 activation; the simultaneous exposure increased intracellular calcium levels, decreased mitochondrial membrane potential, and decreased adenosine-5'-triphosphate (ATP) levels, which resulted in both necrotic and apoptotic cell death (Cunha-Oliveira *et al*., 2010). These two drugs were more toxic when combined, compared to separate administrations. The higher level of cell death that occurred during simultaneous exposure could be related to the presence of COC–MOR adducts.

The mechanisms of action of COC and opioids are different. As already mentioned, the neuropharmacological effects of drugs of abuse are closely related with extracellular DA levels in the *nucleus accumbens*. Both COC and HER increase DA levels in *nucleus accumbens*, but through different mechanisms. HER induces indirect activation of the dopaminergic system by the activation of mu receptors, reducing the inhibitory effect of GABA that is responsible for inhibiting dopaminergic neurones in *nucleus accumbens*; whereas COC increases DA activity by blocking the re-uptake of DA, as aforementioned.

Accordingly, it has been reported that *speedball* induces a synergistic increase of DA in the *nucleus accumbens*, when compared with COC or HER alone (Pattison, McIntosh, Sexton, Childers, & Hemby, 2014). In addition to the greater DA levels in *nucleus accumbens*, a significant decrease in DAT density, as well as an increase in the DAT binding affinity, was observed for *speedball*. Studies demonstrated that DAT apparent affinity is high and similar for COC and *speedball* i.v. administrations, but significantly different from results attained for HER (Pattison, McIntosh, Budygin, & Hemby, 2012). Pattison *et al.* (2012) demonstrated that both chronic COC or *speedball* use increase affinity of low-affinity DAT binding site. So, it is possible that the change on the DAT binding site when COC is consumed alone or in combination might be attributed to COC, and not directly related to *speedball*-induced DA elevations (Pattison *et al.*, 2014).

Of note, these drugs present some opposite effects, which are explained by their action at the noradrenergic system; HER reduces NA activity, while COC increases extracellular NA by blocking its re-uptake at the nerve terminals (Leri *et al.*, 2003; Maldonado, 1997).

When HER and COC are co-used the effects in CNS can be annulated because opioids depress the CNS, while COC stimulates it. On the other hand, their concomitant use can lead to breathing difficulties, since both drugs induce respiratory depression,

increasing the risk of overdose (EMCDDA, 2009). In addition, the simultaneous use of COC can reduce intake of opioids and eliminate physical dependence, as users reveal that they inject equal or higher doses of COC, but less HER, as self-medication to reduce dependence on opioids.

Combination with COC can also affect other properties of opioids. COC use in patients being treated with methadone produces lower blood levels of methadone because COC increases methadone elimination. COC also increases the analgesic efficacy of MOR in rats, mice, and monkeys (Gatch, Negus, Butelman, & Mello, 1995; Hunt, Lipton, Goldsmith, & Strug, 1984; Misra, Pontani, & Vadlamani, 1987; Nott, 1968; Shimada, Tsuda, & Yanagita, 1988; Sierra *et al.*, 1992).

Ward *et al.* (2005) investigated the effects of HER on COC reinforcing effects, using two different self-administration procedures in male rats. Subjects exposed to 1.5 mg COC/kg/infusion presented an average breakpoint (defined as the number of infusions obtained each day) of 17.3±0.67; supplementation with HER (1.5 to 48 µg/kg/infusion) did not produce a significant change on response. Higher doses of COC (0.38 to 3.0 mg/kg/infusion) increased the number of breakpoints, but the addiction of HER (3.0 to 48 µg/kg/infusion) did not significantly changed the observed effects. In this procedure, results on reinforcing effects obtained for all *speedball* combinations tested did not deviate from those attained for COC alone. However, when rats were able to choose, they exclusively opted for COC over HER, and for *speedball* combinations (*e.g.*, 0.18 mg COC/kg/infusion + 50 µg HER/kg/infusion; 0.38 mg COC/kg/infusion + 50 µg HER/kg/infusion) over COC alone (0.75 mg/kg/infusion) (Ward *et al.*, 2005).

In a study performed in males with a recent history of drug abuse, two subjects smoked three increasing doses of HER. The same subjects administered intravenously three increasing doses of the drug, on distinct occasions. Saliva and blood were analysed for the presence of HER and its metabolites (6-MAM and MOR); results are presented in Tables 4 and 5 (Jenkins, Oyler, & Cone, 1995). Robust conclusions regarding the influence of interindividual variability and the administration route on the drug bioavailability were precluded by the absence of standardization of the administered doses among different individuals and among the route of administration. Notwithstanding, from the observed results, it is evident that saliva is a more valuable matrix for detecting smoked HER, while blood must be considered for sampling, when the drug is administered intravenously. HER was detectable in saliva for a longer period than in blood, after smoking the drug; the detection periods in blood and saliva were similar for i.v. administration (Jenkins *et al.*, 1995).

Table 4. Peak concentrations (ng/mL) of parent drug and its metabolites, quantified in saliva (S) and blood (B), after heroin (HER) administration by smoking or intravenous (i.v.) injection in a male volunteer with a recent history of drug abuse (subject A). The S/B ratio is also presented. 6-MAM: 6-Monoacetylmorphine. MOR: Morphine. Adapted from Jenkins et al. (1995).

	Dose administered	HER			6-MAM			MOR		
		Saliva	Blood	S/B	Saliva	Blood	S/B	Saliva	Blood	S/B
Smoking	2.6 mg	3,534	159.00	37.20	1,114	67.80	25.00	76.00	9.10	9.10
	5.2 mg	20,580	51.00	784.30	3,577	21.10	333.60	142.00	4.60	29.40
	10.5 mg	11,700	299.00	39.10	3,510	140.00	36.40	76.00	56.00	4.30
I.V.	5 mg	22.00	42.00	0.91	18.00	32.90	0.71	0	12.70	0
	10 mg	6.00	72.00	0.37	45.00	39.50	7.20	8.00	16.10	0.87
	20 mg	20.00	401.00	0.44	141.00	312.00	5.51	15.00	57.00	1.18

Table 5. Peak concentrations (ng/mL) of parent drug and its metabolites, quantified in saliva (S) and blood (B), after heroin (HER) administration by smoking or intravenous (i.v.) injection in a male volunteer with a recent history of drug abuse (subject B). The S/B ratio is also presented. 6-MAM: 6-Monoacetylmorphine. MOR: Morphine. Adapted from Jenkins et al. (1995).

	Dose administered	HER			6-MAM			MOR		
		Saliva	Blood	S/B	Saliva	Blood	S/B	Saliva	Blood	S/B
Smoking	3.5 mg	4,340	16.70	416.30	750.00	14.00	78.10	37.00	3.30	12.80
	7.0 mg	11,420	52.50	217.50	1,370	14.90	114.10	6.00	29.70	0
	10.5 mg	9,320	108.00	159.90	640.00	55.00	17.40	23.00	9.10	0
I.V.	3 mg	9.00	64.50	0.14	18.00	28.60	1.04	4.00	8.40	0
	6 mg	20.00	315.00	0.45	28.00	126.70	1.78	8.00	105.00	0.98
	12 mg	30.00	141.00	1.90	77.00	151.00	5.26	15.00	44.00	1.82

In this study, seven subjects smoked a single dose of 40 mg of COC. Similarly, they also were intravenously administered 44.8 mg of COC. COC and its metabolites (AEME, BE, BZE, CE, EEE, EME, NBE, NCE, and NCOC) were then quantified in saliva and blood. All subjects who smoked 40 mg of COC presented higher concentrations of COC in saliva, compared to blood; the highest concentrations of COC were detected in saliva 2 min after administration, and ranged from 15,85 to 504,88 ng/mL; these concentrations rapidly declined. Also, the metabolites BZE, EME, NCOC, and AEME were detected, but in lower levels. After the i.v. administration of 44.8 mg of COC, the drug peak concentrations in saliva were lower than those achieved after smoking, ranging from 1.93 ng/mL to 428.00 ng/mL; COC peak concentrations in plasma ranged from 122.00 to 442.00 ng/mL. In conclusion, the saliva is a good biologic matrix for the two routes of COC administration.

Hair is a biological matrix that can be used for a retrospective identification of substances that have short $t_{1/2}$ in other biological matrix, such as blood and urine. In a case report, hair was important to exclude the hypothesis of acetaminophen-codeine ingestion and to ascertain that the baby was chronically exposed to COC and HER (Table 6) (Joya *et al.*, 2011).

Table 6. Toxicological findings in the hair of a baby admitted to the emergency room and of his parents. COC: Cocaine. BE: Benzoylecgonine. MOR: Morphine. 6-MAM: 6-Monoacetylmorphine. Retrieved from Joya et al. (2011).

Specimens	Length of hair (cm)	COC (ng/mg)	BE (ng/mg)	MOR (ng/mg)	6-MAM (ng/mg)	Codeine (ng/mg)
Patient's hair	2	17.5	2.2	2.4	8.1	0.4
Father's hairs	3	11.8	1.7	6.4	8.0	1.8
Mother's hair	12 (total)					
	0-3	2.5	1.6	0.4	0.8	0.1
	3-6	3.7	2.1	0.2	0.6	0.1
	6-9	4.4	4.3	0.2	0.7	0.05
	9-12	3.0	5.5	0.3	0.7	0.1

1.5.3. Cocaine (COC) and ecstasy (MDMA) co-use

Ecstasy is the popular name of 3,4-methylenedioxymethamphetamine (MDMA) , an illicit amphetamine-like substance associated with the rave culture, all-night dance parties occurring during the weekend. Recreational users of MDMA have high incidence of polydrug use, such as consumption of MDMA in combination with amphetamine, methamphetamine or COC (Khorana, Pullagurla, Young, & Glennon, 2004; Williams, Dratcu, Taylor, Roberts, & Oyefeso, 1998; Winstock, Griffiths, & Stewart, 2001). In 2009, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported that more than 50 % of young adults that were COC users also used ecstasy in Cyprus, Ireland, and United Kingdom; while the co-consumption of the these substances rounded 31 % for young COC users in Italy and Portugal(2009).

The co-abuse of MDMA with powdered COC is known as “bumping up”, while “cloud nine” refers to the co-abuse with smoked free-base COC (Khorana *et al.*, 2004). This combination has the intent of intensifying the desired psychostimulant effects of the single drugs (Khorana *et al*., 2004). Despite the number of studies evaluating the toxicity of MDMA and COC alone, there is a lack of information regarding the effects of COC–MDMA co-administration.

Pharmacodynamic interactions may be expected between COC and MDMA, since both drugs share similar mechanisms of action. COC blocks the presynaptic reuptake of DA, NA, and 5-HT, generating an increase in the extracellular levels of these neurotransmitters; whereas MDMA binding to the presynaptic 5-HT transporter (SERT) inhibits the reuptake of 5-HT, which in turn activates DA release by stimulating 5-HT receptors (Muller, Carey, Huston, & De Souza Silva, 2007) and/or by reversing the transporter direction (Crespi, Mennini, & Gobbi, 1997). Evidence suggests that 5-HT is more strongly related to the psychotropic effects of MDMA, compared to COC (Itzhak & Ali, 2006). However, the dopaminergic toxicity induced by MDMA can be affected by the co-abuse of COC.

Panos and Baker (2010) demonstrated that the combination of COC (10 or 20 mg/kg) and MDMA (1.5 or 3.0 mg/kg) increased the extracellular DA levels and the locomotion in rats. Importantly, in this work, the authors vindicated the occurrence of a synergistic effect, as for the mixture administration the measured parameters increased to a greater extent than the increase observed for the administration of either drug alone. Although one must acknowledge this noticeable mixture effect, more caution should be taken by the authors when assuming synergisms, as no expectations for the effects of the combination were provided. Since it is in relation to the additivity calculations that mixture outcomes are evaluated in terms of synergisms (effects greater than additive) or

antagonisms (effects falling short of additivity), conclusions about the type of effect observed are precluded in this study.

Diller *et al.* (2007) reported that in adult male rats co-administration of 5 mg/kg COC and 5 mg/kg MDMA antagonized the rewarding effects of the single drugs, as demonstrated by COC suppression of conditioned place preference (CPP) for MDMA. However, in the same study, it was described that for higher doses of COC and MDMA, the respective drug seeking was reversed. Some research has enlightened that the age of the animals can influence the pre-treatment effect; accordingly, MDMA pre-treatment increased COC-induced CPP in adolescent rats, while decreased it in adults. In this regard, exposure to MDMA during adolescence can render the individuals more prone to polydrug abuse and, therefore, more susceptible to the associated risks, than during adult age (Aberg, Wade, Wall, & Izenwasser, 2007). In mice, exposure to MDMA during adolescence lead to long-lasting neural adaptations to COC reward effects (Achat-Mendes, Anderson, & Itzhak, 2003). Evidence suggested that also differential social and environmental housing conditions can change the response to MDMA in male adolescents rats and increase COC reward (Starosciak, Zakharova, Stagg, Matos, & Izenwasser, 2012).

Additional evidence that MDMA can increase vulnerability to the development of COC dependence was given by Morgan and collaborators (1997) who verified that the pre-treatment of rats with MDMA increased 400% the extracellular levels of DA in nucleus accumbens.

The individual pharmacokinetics of COC and MDMA can also be affected when the drugs are associated, as they are both metabolized by CYP450; therefore, potential toxicodynamic interactions could be expected.

PART II

AIMS

2. AIMS

Over the last 10 years, COC has become the second most used illicit drug in Europe – COC snorting is now the most frequent recreational drug habit after smoking cannabis (EMCDDA, 2012). In 2014, 3.4 million European adults (1% of this population group) have used COC (EMCDDA, 2015). The consumption of this substance became a serious public health concern due to its increasing abuse, but also to its unpredictable acute cardiac and hepatic toxicity, detrimental effects on cognitive functioning, and well-known addictive properties. Similarly to other illicit drugs, COC is all too often taken in recreational contexts combined with other substances. This polydrug abuse pattern hampers toxicological evaluation, as it challenges the establishment of a direct link between detrimental outcomes and a particular drug. In addition, unique random reactions can result from the combination, greatly worsening the associated health risks.

Although COC polydrug abuse is a very common practice, it received rather little attention by the scientific community, when compared with the drug alone. Therefore, this project aimed at increasing the understanding of the toxicological interactions between COC and a few psychoactive agents that are commonly associated with the drug, i.e. HER/ MOR, EtOH, and MDMA. More specifically, the purposes of this work were:

1. To assess whether the cardiotoxic and hepatotoxic effects of mixtures of COC and frequently co-abused substances could be accurately predicted based on the concentration-response data of the individual components, using the models of CA and IA.
2. To classify the mixtures as additive, synergistic or antagonistic by comparing experimental observations with the additivity expectations according to both models.
3. To improve the understanding of the signalling mechanisms underlying the toxicity observed for these mixtures.

PART III

MATERIALS AND METHODS

3. Materials and methods

3.1. Materials

All chemicals used in this study were of analytical grade. Dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), Folin–Ciocalteu reagent, perchloric acid ($HClO_4$), sodium hydroxide (NaOH), cupper (II) sulphate ($CuSO_4$), sodium carbonate (Na_2CO_3), potassium bicarbonate ($KHCO_3$), magnesium sulphate ($MgSO_4$), potassium dihydrogen phosphate (KH_2PO_4), calcium dichloride ($CaCl_2$), EtOH, and disodium phosphate (Na_2HPO_4) were purchased from Merck (Darmstadt, Germany). Sodium phosphate monobasic ($NaHPO_4$) was purchased from Panreac (Barcelona, Spain), potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$) from Fluka (Buchs SG, Switzerland) and sodium chloride (NaCl) was purchased from VWR (Leuven, Belgium). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (Lisbon, Portugal), including COC, HER, and MOR; and all the cell culture reagents were purchased from Gibco (Alfagene, Lisbon, Portugal).

3.2. Animals

Male Wistar Han rats weighting between 200 and 250 g were purchased from Charles-River Laboratories (Barcelona, Spain) and acclimated in polyethylene cages, for at least 2 weeks prior to use, under controlled temperature (20±2 °C), humidity (40–60%) and lighting (12 h light/dark cycle) conditions. The animals had *ad libitum* access to standard rat chow and tap water. On the day of the experiments, the animals were anesthetized with isoflurane prior to the surgical procedures for the isolation of hepatocytes, which were carried out always between 10:00–11:00 a.m. The experiments included in this study were approved by the local committee for the welfare of experimental animals and by the Ethic Commission of the Faculty of Pharmacy of University of Porto, and were performed in accordance with the national Law No. 1005/92, of 23rd October, Article 3, paragraph b, sub-paragraph iii) by investigators accredited by the national authority *Direcção Geral de Alimentação e Veterinária* (DGAV) for laboratory animal use.

3.3. Isolation and culture of hepatocytes

Isolation of hepatocytes was performed by collagenase perfusion, as previously described by Valente *et al*. . Briefly, after perfusion with 600 µM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, a chelation agent that allows the cleavage of the hepatic desmosomes), hepatic collagen was hydrolysed by *ex situ* perfusion

with 100 U/mL *Clostridium histolyticum* collagenase type 1 solution, supplemented with 5.3 µM $CaCl_2$. The hepatocytes were dissociated in Krebs–Henseleit buffer [0.42 M NaCl, 16.9 mM KCl, 4.2 mM $MgSO_4.7H_2O$, 9.1 mM $CaCl_2.2H_2O$, 4.2 mM KH_2PO_4, 25.5 mM $NaHCO_3$, pH 7.4] supplemented with 12.5 mM HEPES and 1% bovine serum albumin (BSA). The obtained hepatocyte suspension was purified by low-speed 5 min-centrifugations at 250 x *g* and incubated for 30 min at 4 °C, with 1% antibiotic solution (10,000 U/mL penicillin; 10,000 µg/mL streptomycin). The initial viability of isolated hepatocytes estimated by the trypan blue exclusion test was always above 85%. A suspension of 5×10^5 viable cells/mL was prepared in complete culture medium, plated at an appropriate cell density, and incubated overnight at 37 °C in a humidified 5% CO_2 – 95 % air atmosphere. The complete cell culture medium consisted of William's E medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 ng/mL insulin solution from bovine pancreas (Sigma-Aldrich, Lisbon, Portugal), 5 nM dexamethasone (Sigma-Aldrich, Lisbon, Portugal), 1% antibiotic solution (10,000 U/mL penicillin; 10,000 µg/mL streptomycin), 10 µg/mL gentamicin, and 0.25 µg/mL amphotericin B.

3.4. H9c2 cell culture

H9c2 cells were generously provided by Dr. Vilma Sardão from The Centre for Neurosciences and Cellular Biology of University of Coimbra, Portugal. The cells were cultured in 75 cm^2 flasks Corning® (VWR, Lisbon, Portugal) with Dulbecco's modified Eagle's medium – high glucose, supplemented with 10% FBS, 1% antibiotic solution (10,000 U/mL penicillin; 10,000 µg/mL streptomycin) and 1% MEM non-essential amino acids (NEAA). Cells were maintained at 37 °C in a humidified 5% CO_2 – 95 % air atmosphere and fed every 2–3 days. When the flasks reached 70–80% confluence, the cell culture medium was removed and cells were washed with pre-warmed Hank's balanced salt solution (HBSS); then cultures were passaged by trypsinization (0.25% trypsin solution with 1 mM EDTA) and were sub-cultured over a maximum of 10 passages.

3.5. Mixture testing

COC was combined with HER (Mix A; HER 2: COC 1), MOR (Mix B; MOR 2: COC 1), EtOH (Mix C; EtOH 9: COC 1), or MDMA (Mix D; MDMA 5: COC 1) at realistic mixture ratios, *i.e.* at the same mixture ratio found in the blood of intoxicated abusers (Jenkins, Levine, Titus, & Smialek, 1999) or in seized *ecstasy* pills (information retrieved from http://www.ecstasydata.org/view.php?id=3554 accessed on July 5, 2016).

3.6. Drug exposures

After seeding, primary rat hepatocytes and H9c2 cells were incubated overnight at 37 °C with 5% CO_2 for cell adhesion. In the next day, cells were exposed to the drugs and their mixtures in complete cell culture medium for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay was performed both in primary and immortalised cells to evaluate cytotoxicity elicited by treatments. Primary hepatocytes were exposed to COC, EtOH, HER, MDMA, Mix A, Mix C, and Mix D; while H9c2 cells were exposed to COC, EtOH, MOR, MDMA, Mix B, Mix C, and Mix D. All these treatments were tested at a large range of concentrations, so that complete concentration–response relationships could be recorded.

Besides the MTT viability assay, additional assays were performed in H9c2 to clarify the underlying mechanisms of cardiototoxicity induced by COC, EtOH, MOR, MDMA, Mix B, Mix C, and Mix D. These experiments were conducted by testing all treatments at the respective concentrations producing 30% and 60% of the maximal effect in the viability assay (EC_{30} and EC_{60}, respectively) – these effect concentrations were estimated on the basis of the concentration-response curves obtained for the corresponding treatments in the MTT assay.

All stock solutions of the testing drugs and their mixtures were made in HBSS, stored at -20 °C, and freshly diluted on the day of the experiment. By employing *the fixed mixture ratio design* (Altenburger *et al*., 2000), a range of concentrations of each mixture was subsequently prepared for testing, maintaining the ratio between each constituent unchanged. Positive and negative controls (complete cell culture medium) were performed parallel to drug incubations.

3.7. Determination of cell viability

The MTT assay assesses cell viability indirectly through the measurement of activity of enzymes that reduce the yellow soluble MTT to purple insoluble formazan salts. As MTT can only be reduced when mitochondrial reductases are active, the conversion is used as a measure of the organelle viability and, therefore, of cell viability. The purple formazans are dissolved by DMSO, and the absorbance of the coloured solution quantified at 550 nm.

For the viability assay, H9c2 cells and primary rat hepatocytes were seeded at a density of ~2×10^4 cells/cm^2 and ~1.5×10^5 cells/cm^2, respectively, onto the central 60 wells of the 96-well plates (BD Falcon, Enzifarma, Lisbon, Portugal). After 24h-incubations with the testing drugs, the cell culture medium was aspirated from the plate and 100 µL of 1 mg/mL MTT (Sigma-Aldrich, Lisbon, Portugal) solution was added to each well. The cells

were further incubated at 37°C, for 4h (H9c2 cells) or 1h (rat hepatocytes). Then, the MTT solution was aspirated and the formed crystals were dissolved with 100 µL of DMSO. The plate was shaken for 15 min, protected from light. The absorbance was measured at 550 nm, directly in the plate, using a multi-well plate reader BioTek Synergy™ HT (BioTek Instruments, Inc.).

All steps of the procedure were executed under the light protection because MTT is photosensitive. The results were normalised with negative and positive (1% Triton X-100) controls and results were graphically presented as percentage of cell death *versus* concentration (mM).

3.8. Prediction of mixture effects

The expected mixture effects were calculated using two pharmacological models of additivity that are widely employed in the mixture toxicity field, *concentration addition* (CA) and *independent action* (IA).

The foundations of CA model are that mixture constituents act by a similar mode of action, which means that any component contributes to the global effect in proportion to its concentration, even when present at concentrations that do not produce effect. Presuming that the effect of a mixture with *n* components is concentration additive, the mixture effect is calculated by $ECmix = [\sum pi/ECi]^{-1}$, where *ECmix* is the mixture concentration eliciting an effect of defined magnitude, *pi* is the fraction of each individual compound relative to the total mixture concentration that is required to produce effect *i*, and *ECi* is the concentration of each individual compound that can produce effect *i*.

It is preconized that the IA model is better applied to mixtures of drugs with different mechanisms of action or interacting at differing sites. Thus, IA assumes that mixture components present at zero effect concentrations will not contribute to the overall effect because the fractional response of a single component is supposed to be independent of those induced by other components. The mixture effect might be estimated by $Emix = Emax[1 - \prod[1 - Fi(ci)/Emax]]$, where *Emax* is the maximal measured effect, and *Fi(ci)* the mean effect predicted by the regression model for each individual drug.

3.9. Samples preparation for determination of adenosine-5'-triphosphate (ATP) and reduced (GSH) and oxidized glutathione (GSSG)

H9c2 cells were seeded onto 6-well plates at a density of ~2×10^4 cells/cm^2, and incubated overnight. Then, cells were incubated with the testing drugs and, after 24h, the exposure medium was collected into a centrifuge tube. The cells were washed twice with 1

mL of HBSS with Ca and Mg, and the washing buffer was also collected into the centrifuge tube containing the exposure medium. After centrifugation for 10 min at 16,000 x *g* in a refrigerated centrifuge (4 °C), the supernatant was rejected, and the pellet kept on ice. The cells were added of 300 µL 5% $HClO_4$ and the plate was kept on ice, for 20 min. After scrapping, cell suspension was transferred into the centrifuge tube placed on ice; the cell pellet was resuspended and sample centrifuged at 4 °C for 10 min at 16,000 x *g*. The supernatant was collected into a clear microcentrifuge tube and kept at -80 °C until further determination of ATP, reduced glutathione (GSH) and oxidized glutathione (GSSG). The pellet was resuspended in 0.3 M NaOH and used for protein quantification.

3.10. Determination of reduced (GSH) and oxidized glutathione (GSSG)

The quantification of total GSH (GSHt) in H9c2 cells was performed through the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)-GSSG reductase-recycling assay. Briefly, after neutralizing the supernatants of samples, blank and standards with 0.76 M $KHCO_3$ (until there was no formation of CO_2 following vortex mixing), the microcentrifuge tubes were centrifuged for 10 min at 16,000 x *g* (4 °C). Then, 100 µL of each supernatant were added to a 96-well plate, followed by 65 µL of a freshly prepared reagent solution containing 0.24 mM NADPH and 0.7 mM DTNB, prepared in a phosphate buffer (71.5 mM $NaHPO_4$, 71.5 mM Na_2HPO_4, and 0.63 mM EDTA, pH 7.0). Samples were incubated for 15 min at 30 °C. Then, 40 µL of 10 U/mL glutathione reductase (GR, Sigma-Aldrich) solution (freshly prepared in phosphate buffer) were added to each well. The consequent formation of 5-thio-2-nitrobenzoic acid (TNB) was followed for 3 min, every 10 sec, at 405 nm, using a multi-well plate reader (Power Wave X^{TM}, BioTek Instruments, Inc.).

To determine intracellular GSSG, 10 µL of 2-vinylpyridine (Sigma-Aldrich) were added to 200 µL of neutralised supernatants (samples, blank, and standards) and mixed for 1h, on ice. As 2-vinylpyridine blocks GSH, GSSG will be determined as described for GSHt. The amount of GSH was calculated by subtracting GSSG from the GSHt, *i.e.* GSH=GSHt–(2xGSSG).

The GSSG and GSH standard solutions were prepared in 5% $HClO_4$; the concentrations ranged between 0.10 and 13 µM for GSSG, and between 0.33 and 13 µM for GSH. Results were compared with the respective standard curve, normalised to the amount of protein, and presented as nmol per mg of protein.

3.11. Determination of adenosine-5'-triphosphate (ATP)

The intracellular level of ATP was determined through the luciferin–luciferase bioluminescence assay that measures the light formed in the oxidation of luciferin, catalysed by luciferase in the presence of ATP. The amount of ATP is directly proportional to the emitted light intensity (maximum at ~560 nm, pH 7.8).

After neutralizing the supernatants of samples, blank, and standards with 0.76 M $KHCO_3$ (until there is no formation of CO_2 following vortex mixing), the microcentrifuge tubes were centrifuged for 10 min at 16,000 x *g* (4 °C). After that, 75 µL of each supernatant (4 °C) was transferred into a 96-well plate and added of 75 µL of luciferin-luciferase reagent (4 °C, protected from light), immediately before the luminescence readings. Results were compared with the standard curve, normalised to the amount of protein, and presented as nmol ATP per mg of protein. The ATP (Sigma-Aldrich) standard solutions were prepared in 5% $HClO_4$; the concentrations ranged between 1.25 and 20 µM.

3.12. Determination of protein

The protein content in the samples was measured through the method of Lowry (1951). Accordingly, 50 µL of each sample, standard, or blank were transferred into a 96-well plate, in triplicate, and added of 100 µL reagent A (14.7 mL of 2% Na_2CO_3, 0.15 mL of 2% $KNaC_4H_4O_6.4H_2O$, and 0.15 mL of 1% $CuSO_4.5H_2O$, extemporaneously prepared). The plate was incubated in the dark for 10 min, at room temperature, followed by the addition of 100 µL of reagent B (Folin and Ciocalteu's phenol reagent, diluted 15x in purified water). The plate was incubated for 20 min, under light protection, at room temperature, and the absorbance was measured at 750 nm in a 96-well microplate reader (Power Wave X™, BioTek Instruments, Inc.). Protein standards were prepared with BSA in 0.3 M NaOH (concentrations between 31.25 µg/mL and 500 µg/mL).

3.13. Measurement of reactive species

Measurement of intracellular ROS and RNS was made by a fluorescent assay that uses 2',7'-dichlorofluorescin diacetate (DCFH-DA), a non-fluorescent lipophilic compound that passes through the cell membrane, being deacetylated to 2',7'-dichlorofluorescin (DCFH) by intracellular esterases. DCFH is a sensitive probe that reacts with intracellular ROS and RNS to generate a green fluorescent polar compound, 2',7'-dichlorofluorescein (DCF), which is trapped within the cells.

In our experiments, H9c2 cells were seeding onto 96-well plates at a density of ~2×10^4 cells/cm^2. Before drug exposures, the cell culture medium was gently aspirated,

cells were washed twice with HBSS and incubated with 100 µL of 10 µM DCFH-DA (Sigma-Aldrich) for 30 min, at 37 °C. At the end of the incubation period, cells were washed twice with HBSS and added of 100 µL of testing solutions. Following incubation with the treatments at 37 °C for 24h, fluorescence was read in a multi-well plate reader (BioTek Synergy™ HT) set to 485 nm excitation and 530 nm emission. As DCFH-DA is photosensitive, all steps of the procedure were executed under light protection.

3.14. Evaluation of mitochondrial integrity (Δψm)

Mitochondrial integrity was assessed by measuring the tetramethylrhodamine ethyl ester perchlorate (TMRE) inclusion. This cell permeable fluorescent dye specifically stains live mitochondria, accumulating in proportion to the mitochondrial membrane potential (Δψm).

The H9c2 cells were seeding onto 96-well plates at a density of ~2×10^4 cells/cm^2 and incubated at 37°C overnight. On the day of the experiment, cells were exposed to the drugs for 24h. Then, the exposure medium was gently removed, the cells were washed twice with HBSS, added of 100 µL of 2 µM TMRE (Sigma-Aldrich), and incubated for 30 minutes at 37 ^oC. Cells were washed twice and added of 100 µL of HBSS. The fluorescence was read in a multi-well plate reader (BioTek Synergy™ HT) set at 544 nm excitation and 590 nm emission.

3.15. Determination of cell membrane integrity

Cytoplasmic membrane integrity was evaluated through the lactate dehydrogenase (LDH) leakage assay. As LDH is a cytoplasmic oxidoreductase, its presence in the extracellular medium is indicative of alterations in membrane permeability and consequently in cell integrity. The enzyme catalyses the reversible conversion of pyruvate to lactate, in the presence of nicotinamide adenine dinucleotide (NADH), which by turn is oxidized to NAD+.

H9c2 cells were seeding into a 96-well plate at a density of ~2×10^4 cells/cm^2. At the end of the incubation period, 50 µL of supernatant was transferred into a 96-well plate and added of 200 µL of freshly prepared 0.15 mg/mL β-nicotinamide adenine dinucleotide (β-NADH) solution. Immediately before the absorbance reading, 25 µL of 2.5 mg/mL sodium pyruvate solution were added to start the reaction. Both β-NADH and sodium pyruvate were prepared in potassium phosphate buffer (KH_2PO_4, pH 7.4). The kinetic of the oxidation of NADH to NAD^+ was followed by reading the absorbance at 340 nm, every 16 seconds, for 3 minutes, using an automatic plate reader Power Wave XTM (BioTek Instruments, Inc.).

PART IV

RESULTS

4. Results

4.1. Mixture effects of cocaine (COC) and other drugs frequently co-abused

For the accurate calculation of the effects of a given mixture, besides knowledge on its exact composition, information on the effects of all single drugs is required. Therefore, to ensure great precision of our predictions, the utilisation of a highly reproducible assay is demanded. Moreover, this has to be able to produce large amounts of highly reliable data for every component of the mixture, in a relatively short period of time. Accordingly, the MTT reduction assay was selected to evaluate the effects of different mixtures of COC on cell viability, in two different cell models, *i.e.* hepatocytes and cardiomyocytes. The data attained was then used for the assessment of the type of interaction (synergism, additivity, or antagonism) that occurred between COC and MOR (Mix B), COC and EtOH (Mix C), and COC and MDMA (Mix D), in H9c2 cells; and between COC and HER (Mix A), COC and EtOH (Mix C), and COC and MDMA (Mix D), in primary rat hepatocytes.

4.1.1. Hepatotoxicity elicited by cocaine (COC) combined with heroin (HER), ethanol (EtOH), or *ecstasy* (MDMA)

The assessment of combination effects in terms of synergisms, antagonisms, or additivity critically depends on the determination of the expected effects of a given mixture, and on the subsequent comparison of these calculations with the mixture effects experimentally attained. As already mentioned, in order to produce the data required for calculating predictions of mixture effects, extensive concentration–response analyses of all individual mixture components had to be carried out. Therefore, we first analysed the mortality elicited by COC, HER, EtOH, and MDMA alone, after exposures of primary hepatocytes for 24 h.

As observed in Figure 5 in primary hepatocytes, all single drugs produced complete mortality profiles, *i.e.* concentration-dependent responses ranging from 0% to 100% cell death. The presented data were produced in four different occasions, using independently prepared serial dilutions of all chemicals; there was always good agreement between experiments. Regarding the cytotoxic potency, EtOH (EC_{50} 263.26 mM) was less toxic than COC (EC_{50} 1.07 mM), MDMA (EC_{50} 0.56 mM), and HER (EC_{50} 0.26 mM). In what concerns the slope, MDMA was the drug presenting the steepest curve. A summary of the best-fit regression model parameters (including location and slope) and the concentrations that individually produce 50% of the maximal effect (EC_{50}) for each drug are presented in Table 7.

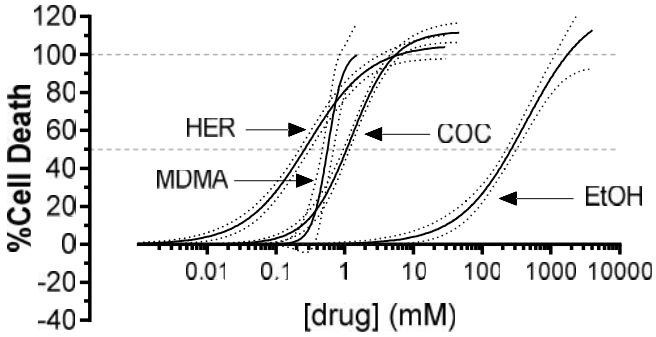

Figure 5. Cytotoxicity elicited by cocaine (COC), heroin (HER), ethanol (EtOH), and ecstasy (MDMA) in primary rat hepatocytes, after 24 h-exposure, at 37 °C. Data assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay are from four independent experiments (in triplicates) and are presented as percentage of cell death (relative to the negative controls). The solid lines are the best estimate of mean responses given by the dosimetric Logit model, and the dotted lines are the respective upper and lower limits of the 95% confidence interval. The dashed grey lines represent 50% and 100% effect.

After thoroughly characterize the concentration–effect relationships of individual drugs in terms of shape, slope, and maximal effects (Table 7), the CA and IA models were applied to quantitatively fit the expected responses of three different mixtures. As observed in Figure 6, in primary rat hepatocytes, the models CA and IA predicted roughly coincident toxicity for both Mix A (EC_{50} 0.37 mM and 0.37 mM, respectively), Mix C (EC_{50} 9.02 mM and 9.03 mM, respectively), and Mix D (EC_{50} 0.61 mM and 0.66 mM, respectively) (Table 8).

Effects experimentally obtained for Mix A (EC_{50} 0.53±0.07 mM) were shifted to the right, compared to predictions; for Mix C, were shifted to the left (EC_{50} 5.60±0.30 mM); while for Mix D, the effects calculated by CA were comprised in the 95% confidence belt of the observed effects (EC_{50} 0.55±0.10 mM). Consequently, the toxicity observed for Mix C was higher than the calculated, indicating the occurrence of synergism between EtOH and COC; the opposite occurred for Mix A, *i.e.* antagonism between COC and HER; while for Mix D, predicted and observed toxicities were coincident suggesting that effects of COC and MDMA are additive.

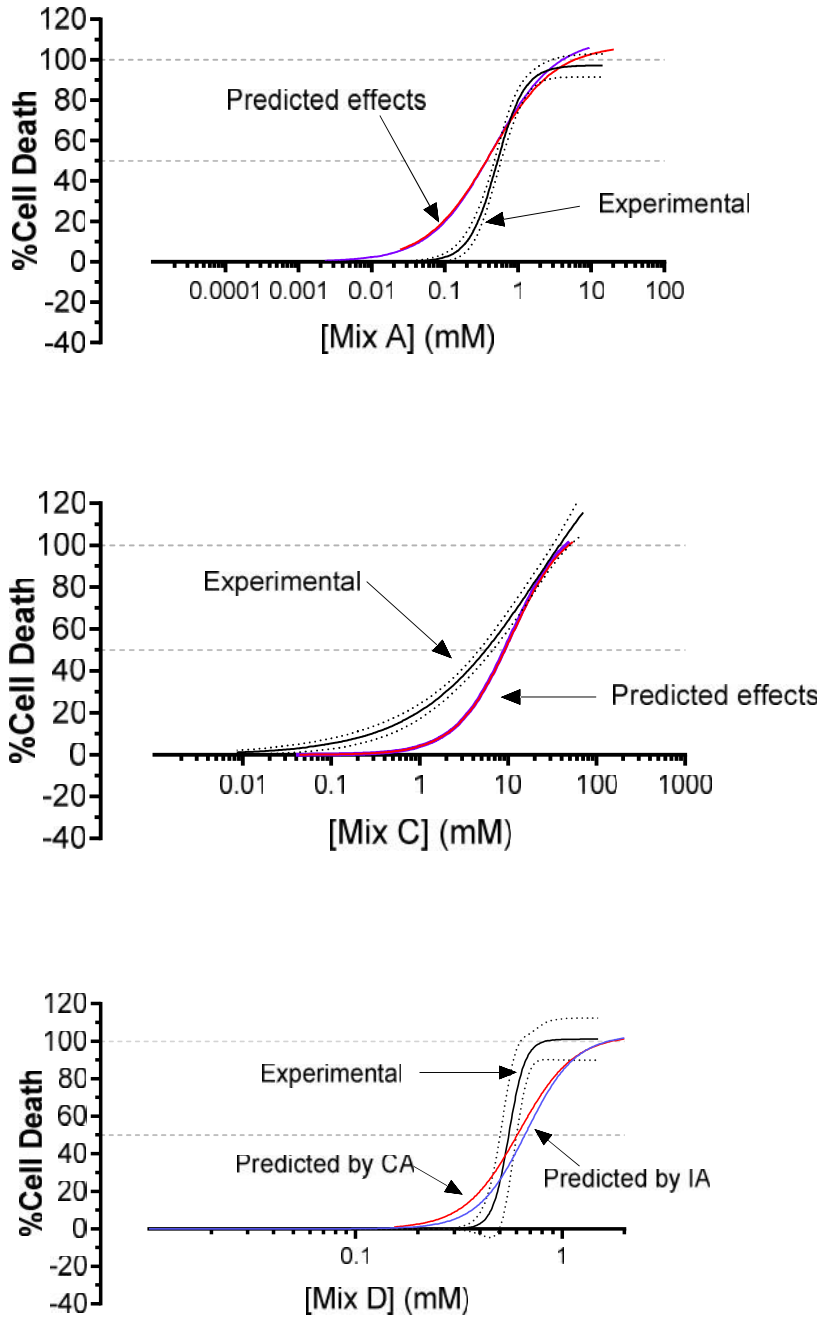

Figure 6. Mortality elicited by the mixtures of cocaine 1: heroin 2 (Mix A), cocaine 1: ethanol 9 (Mix C), and cocaine 1: MDMA 5 (Mix D), in primary rat hepatocytes, as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, after 24h of exposure, at 37 °C. Purple and red lines: mixture effects predicted by independent action (IA) and concentration addition (CA), respectively. Black lines: experimental mixture effects. Data are presented as percentage of cell death relative to the negative controls and are from a minimum of four independent experiments, performed in triplicate. Curves were fitted to the dosimetric Logit model (Table 7). The dotted lines are the upper and lower limits of the 95% confidence interval of the best estimate of mean responses. The dashed grey lines represent 50% and 100% effect.

4.1.2. Cardiotoxicity elicited by cocaine (COC) combined with morphine (MOR), ethanol (EtOH), or *ecstasy* (MDMA)

Similar to results obtained for primary hepatocytes, all single drugs, *i.e.* COC, MOR, EtOH, and MDMA, yielded reproducible concentration-dependent toxicity, when tested in H9c2 cells. The cytotoxicity curves for each of the testing drugs, including the upper and lower 95% CI, are displayed in Figure 7. Also, all drugs produced complete curves of percentage of cell death *versus* drug concentration. Differences were observed essentially in the EC_{50} values and in the slopes. Accordingly, EtOH with an EC_{50} of 305.26 mM was the least toxic drug, followed by MOR (EC_{50} 6.93 mM) and COC (EC_{50} 2.60 mM). Contrarily, MDMA was the most cardiotoxic drug (EC_{50} 1.07 mM). These differences between EC_{50} values of the testing drugs were deemed statistically significant, as there was no overlap between the corresponding 95% CI of the concentration–response curves (Figure 8).

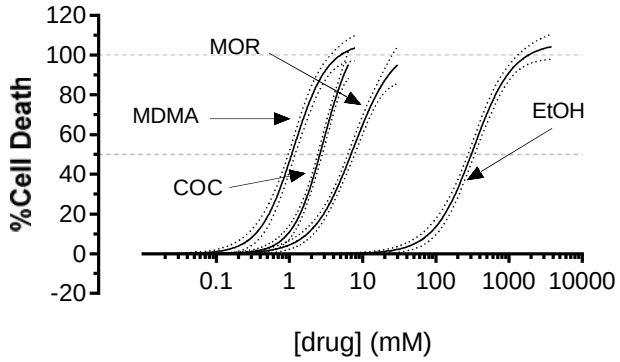

Figure 7. Cytotoxicity elicited by cocaine (COC), morphine (MOR), ethanol (EtOH), and ecstasy (MDMA), in H9c2 cells, after 24h-exposure, at 37 °C. Data assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay are from four independent experiments (in triplicates) and are presented as percentage of cell death (relative to the negative controls). The solid lines are the best estimate of mean responses given by the dosimetric Logit model, and the dotted lines are the respective upper and lower limits of the 95% confidence interval. The dashed grey lines represent 50% and 100% effect.

Using the data attained for COC, MOR EtOH, and MDMA in H9c2 cells, mixture toxicity was predicted by CA and IA for Mix B (EC_{50} 4.18 mM and 5.01 mM, respectively), Mix C (EC_{50} 21.75 mM and 22.95 mM, respectively), and for Mix D (EC_{50} 1.19 mM and 1.28 mM, respectively) (Figure 8 and Table 8). Again, calculations from both models were congruent, but CA revealed to be slightly more conservative (as EC_{50} values were marginally smaller than those achieved for IA).

Experimental mixture testing at the conditions used for the individual drugs demonstrated that, in H9c2 cells, the predicted toxicity was lower than the effects

experimentally attained for mix B (EC_{50} 1.90±0.23 mM) and mix D (EC_{50} 0.41±0.20 mM) (synergism) and roughly similar for mix C (EC_{50} 19.18±3.36 mM) (additivity).

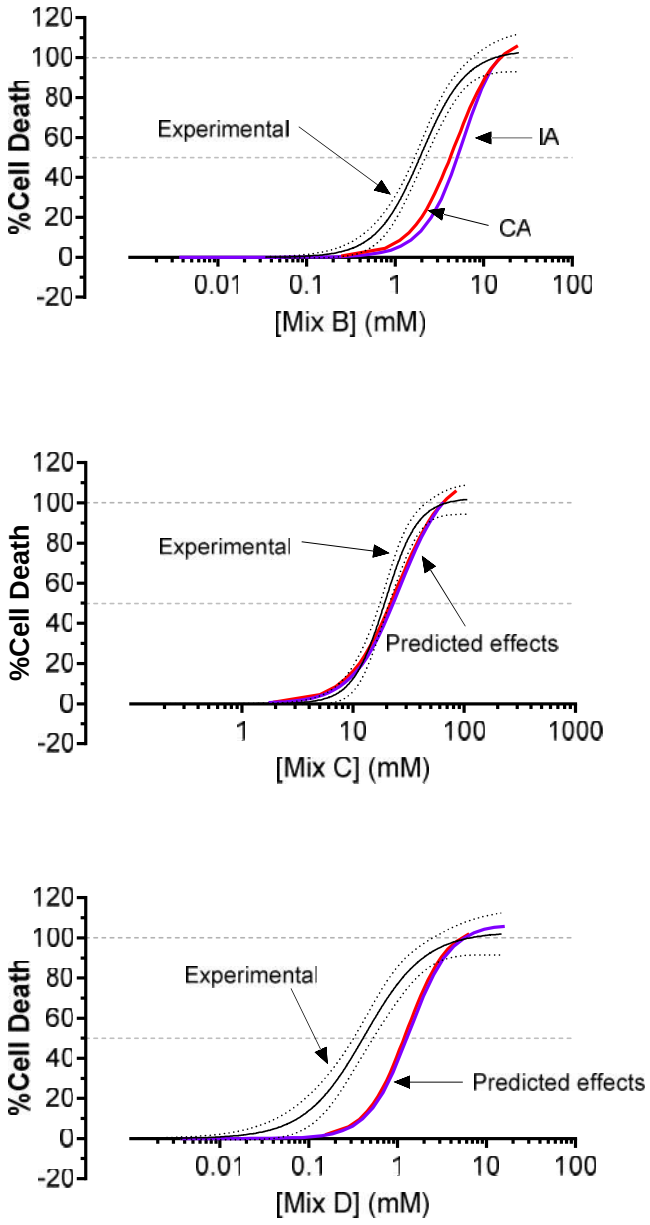

Figure 8. Mortality elicited by the mixtures of cocaine 1: morphine 4 (Mix B), cocaine 1: ethanol 9 (Mix C), and cocaine 1: MDMA 5 (Mix D), in H9c2, as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, after 24h of exposure, at 37 °C. Purple and red lines: mixture effects predicted by independent action (IA) and concentration addition (CA), respectively. Black lines: experimental mixture effects. Data are presented as percentage of cell death relative to the negative controls and are from a minimum of four independent experiments, performed in triplicate. Curves were fitted to the dosimetric Logit model (Table 7). The dotted lines are the upper and lower limits of the 95% confidence interval of the best estimate of mean responses. The dashed grey lines represent 50% and 100% effect.

Table 7. Parameters derived from the nonlinear fits of the single drugs. Concentration–mortality data from the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, obtained after 24h-incubations at 37 ^{o}C, were fitted to the asymmetric Logit function. These parameters were used to compute the mixture effects, shown in Figures. 6 and 8. Mix A – mixture of cocaine (COC) and heroin (HER) at 1:2. Mix B – mixture of COC and morphine (MOR) at 1:2. Mix C – mixture of COC and ethanol (EtOH) at 1:9. Mix D – mixture of COC and ecstasy (MDMA) at 1:5

	Estimated parameters for the regression model			**EC_{50}**	**Fraction in mixture**			
	Θ_1[A]	Θ_2[B]	Θ_{MAX}[C]		**Mix A**	**Mix B**	**Mix C**	**Mix D**
			Primary hepatocytes					
COC	-0.31	3.24	112.20	1.07	0.38	-	0.11	0.17
HER	1.20	2.21	105.20	0.26	0.62	-	-	-
EtOH	-5.56	2.12	127.00	263.26	-	-	0.89	-
MDMA	-6.67	8.84	102.00	0.56	-	-	-	0.83
Mix A	1.53	5.25	97.27	0.52	1.00	-	-	-
Mix C	-1.99	1.47	172.1	5.60	-	-	1.00	-
Mix D	-18.90	25.49	101.10	5.52	-	-	-	1.00
			H9c2 cells					
COC	-2.24	4.80	113.90	2.60	-	0.38	0.11	0.17
MOR	-3.13	3.59	105.90	6.93	-	0.62	-	-
EtOH	-9.37	3.72	106.10	305.26	-	-	0.89	-
MDMA	-0.25	4.33	106.3	1.07	-	-	-	0.83
Mix B	-1.17	3.93	103.80	1.91	-	1.00	-	-
Mix C	-8.78	6.81	102.30	19.18	-	-	1.00	-
Mix D	1.08	2.5	103.00	0.41	-	-	-	1.00

a Location parameter of the Logit function.
b Slope parameter.
c Maximal effect, expressed as corrected % cell death.

Table 8. Observed and predicted EC_{50} values for the tested combinations, i.e. mixture A (cocaine 1: heroin 2), mixture B (cocaine 1: morphine 4), mixture C (cocaine 1: ethanol 9), and mixture D (cocaine 1: ecstasy 5) in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, after 24h-incubations at 37 °C.

Cell model	Mixture	EC_{50} (mM)			Combination effect
		Predicted by CA	Predicted by IA	Observed	
Primary rat hepatocytes	A	0.366	0.369	0.525±0.074	Antagonism
	C	9.019	9.034	5.601±0.302	Synergism
	D	0.606	0.660	0.552±0.102	Additivity
H9c2 cardiomyocytes	B	4.180	5.005	1.903±0.229	Synergism
	C	21.753	22.946	19.183±3.360	Additivity
	D	1.186	1.277	0.410±0.195	Synergism

4.2. Mechanisms underlying the observed joint effects

In order to obtain an in-depth understanding of the pathways underlying the observed toxicological effects, the three mixtures tested in H9c2 were further investigated. Accordingly, alterations on a set of parameters related to mechanisms of oxidative and mitochondrial stress were evaluated, specifically the intracellular contents of GSH, GSSG, ATP, and ROS/RNS, and alterations on the mitochondrial membrane potential. Two concentrations, corresponding to the respective EC_{30} and EC_{60}, of each drug and their mixtures were tested; these concentrations were estimated from the Logit regression model of each data set obtained in the MTT assay (Table 9).

Table 9. Concentrations (mM) used for evaluating the mechanisms underlying cardiotoxicity of cocaine (COC), morphine (MOR), ethanol (EtOH), and ecstasy (MDMA) when H9c2 were exposed to the drugs alone or in combination (i.e. Mix B, Mix C, and Mix D), for 24 h at 37 °C. These concentrations correspond to the respective EC_{30} and EC_{60}, as obtained in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Mix B: mixture of COC 1: MOR 2. Mix C: mixture of COC 1: EtOH 9. Mix D: mixture of COC 1: ecstasy (MDMA) 5.

	EC_{30}	EC_{60}
COC	1.788	3.085
MOR	4.105	8.841
EtOH	184.338	385.781
MDMA	0.694	1.308
MIX B	1.172	2.388
MIX C	14.465	21.921
MIX D	0.215	0.557

By neutralising intracellular reactive species, GSH is capable of preventing damage to vital cellular components, such as proteins, lipids, and DNA; therefore, changes in the intracellular contents of GSH and GSSG are important indicators of redox disturbance. Our data from quantification of intracellular amounts of GSH and GSSG demonstrate that all treatments disturbed thiol homeostasis, in H9c2 cells after 24 h-exposure, particularly, at the highest concentration tested (EC_{60}) ($p<0.01$, ANOVA/Holm-Sidak's). The exception was EtOH; although a decrease in tGSH was observed for both concentrations, it did not reach statistical importance ($p>0.05$, ANOVA/Holm-Sidak's). These changes on the intracellular tGSH levels were strictly correlated with alterations on GSH contents (Figure 9). Also, important modifications on intracellular GSSG were observed; these were more pronounced for COC, when tested at the EC_{60} ($p<0.05$, ANOVA/Holm-Sidak's). Moreover, this result was significantly different from that produced by mixtures B and C, at the same concentration ($p<0.05$, ANOVA/Holm-Sidak's). Also MOR at EC_{60} and COC at EC_{30} yield tGSH results that deviated from those obtained for Mix B and Mix C, respectively, tested at the same concentrations ($p<0.05$, ANOVA/Holm-Sidak's).

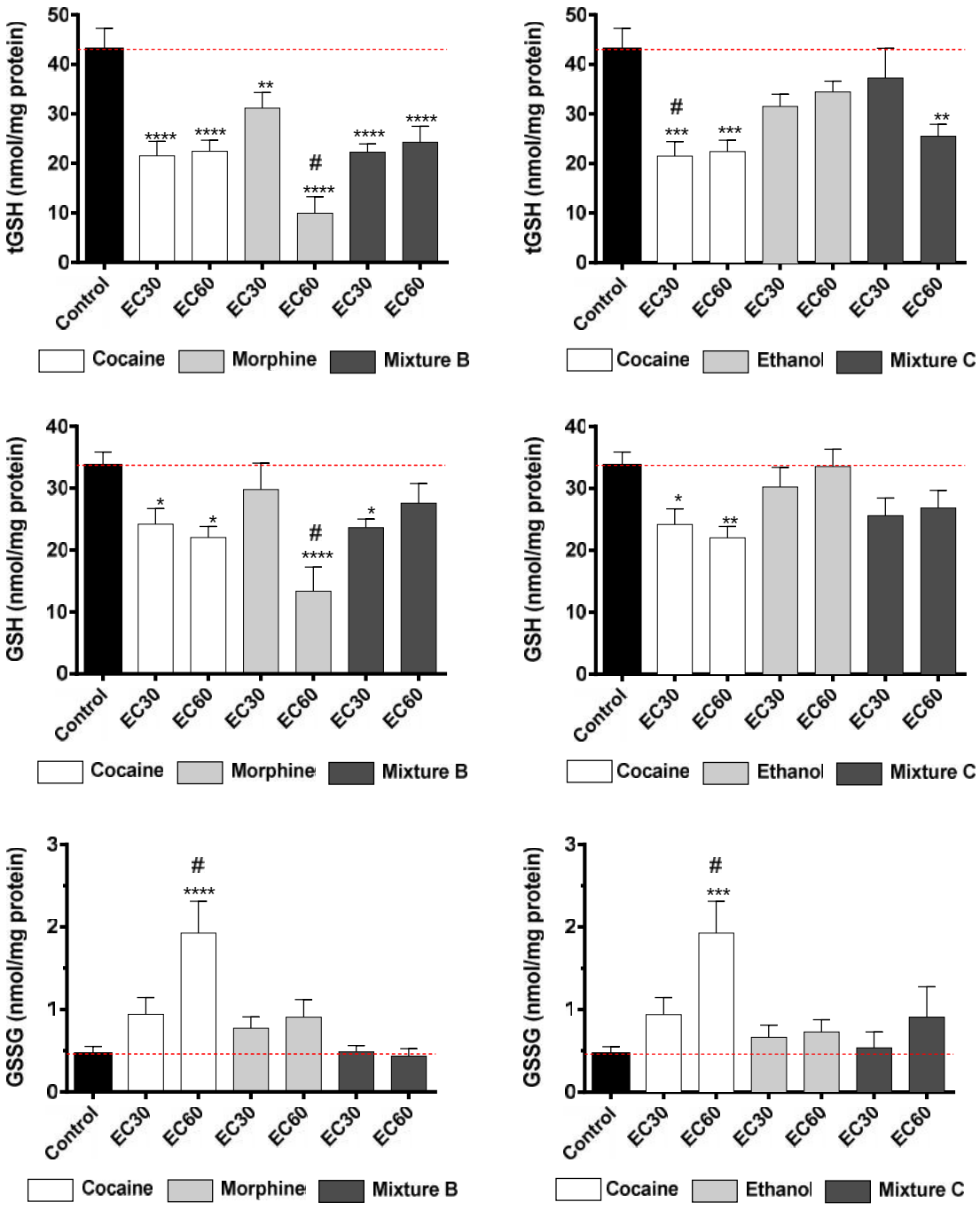

*Figure 9. Effect elicited by cocaine, morphine, ethanol, and mixtures B and C in the total glutathione (tGSH), reduced glutathione (GSH), and glutathione disulfide (GSSG), in H9c2 cells, after 24 h-exposure at 37 ºC. EC_{30} and EC_{60} are the concentrations producing 30% and 60% of effect, respectively, in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. Mixture B: mixture of COC 1: MOR 2. Mixture C: mixture of COC 1: EtOH 9. Results normalized to the protein content represent the mean ± SEM. Statistical comparisons were made by one-way ANOVA followed by Holm-Sidak's multiple comparison post hoc test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared to controls. #p<0.05, compared to mixture tested at the same concentration.*

Thiol antioxidant stores protect cells against oxidative injury, but the disruption of glutathione homeostasis will render the cells vulnerable to the stress induced by free

radicals. Accordingly, the results obtained in the DCFH-DA assay confirmed that all treatments stimulated the production of ROS/RNS after 24 h-exposures, in H9c2 (Figure 10); these results were significant at the highest concentration tested (p<0.0001, ANOVA/Holm-Sidak's). Remarkably, Mix D, MDMA, and EtOH also induced significant production of prooxidant species at the lowest concentration (EC_{30}; p<0.05, *vs.* control; ANOVA/Holm-Sidak's). Similarly to results of GSH assay, COC and Mix C were significantly different, when tested at the respective EC_{60} (p<0.05, ANOVA/Holm-Sidak's).

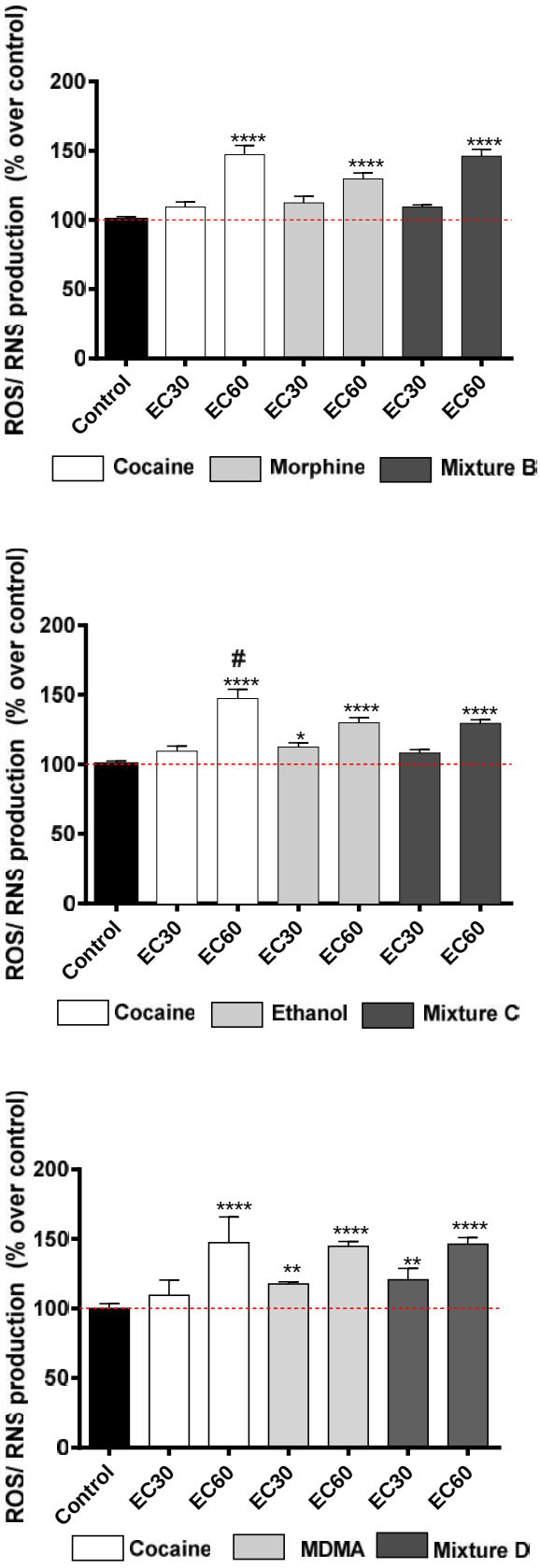

Figure 10. Reactive species of oxygen (ROS) and nitrogen (RNS) formed in H9c2, after exposure at cocaine, morphine, ethanol, and mixtures B and C, for 24 h at 37°C. EC_{30} and EC_{60} are the concentrations producing

*30% and 60% of effect, respectively, in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. Mixture B: mixture of COC 1: MOR 2. Mixture C: mixture of COC 1: EtOH 9. Mixture D: mixture of COC 1: ecstasy (MDMA) 5. Results represent the mean ± SEM of the fluorescence emitted when 2',7'-dichlorodihydrofluorescein (DCFH) reacts with reactive species, and were normalized to the controls. Statistical comparisons were made by one-way ANOVA followed by Holm-Sidak's multiple comparison post hoc test. *p<0.05, ****p<0.0001, compared to controls. #p<0.05, compared to mixture tested at the same concentration.*

Mitochondria is a major site for ROS/RNS production and a strong positive correlation between ΔΨm and ROS production has been recurrently demonstrated, as mitochondria produce more ROS at high membrane potential (Turrens, 2003). In accordance, our data indicates that all treatments induced a significant increase of ΔΨm (p<0.01, ANOVA/Holm-Sidak's), measured through TMRE incorporation in mitochondria of H9c2 cells (Figure 11). MORPH tested at EC_{30} was the only treatment that deviated from mixture results (p<0.05 *vs.* EC_{30} Mix B, ANOVA/Holm-Sidak's).

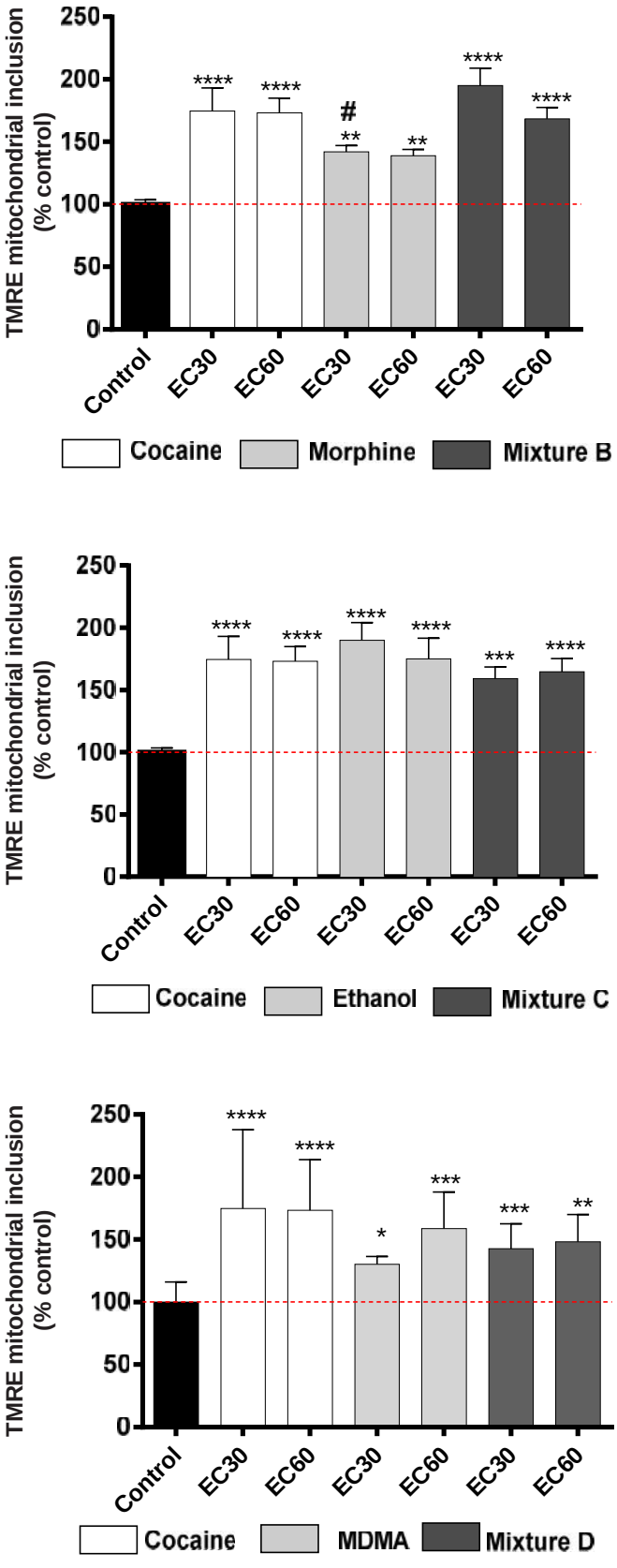

Figure 11. Effect elicited by cocaine, morphine, ethanol, and mixtures B and C on the amount of tetramethylrhodamine ethyl ester perchlorate (TMRE) incorporated within the mitochondria, as a measure of

*mitochondrial membrane potential, in H9c2 cells, after 24 h-exposure. EC_{30} and EC_{60} are the concentrations producing 30% and 60% of effect, respectively, in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. Mixture B: mixture of COC 1: MOR 2. Mixture C: mixture of COC 1: EtOH 9. Mixture D: mixture of COC 1: ecstasy (MDMA) 5. Results were normalized to the controls and presented as mean ± SEM. Statistical comparisons were made by one-way ANOVA followed by Holm-Sidak's multiple comparison post hoc test. **p<0.01, ***p<0.001, ****p<0.0001 compared to controls. #p<0.05, compared to mixture tested at the same concentration.*

Mitochondrial membrane potential is critical for preserving the functional integrity of the respiratory chain and, therefore, of ATP generation. An increase in the ΔΨm has been related to inhibition or dysfunction of ATP synthase (Geromel *et al*., 2001; Wojtczak *et al.*, 1999), with parallel increase of ROS production. In line with these observations, all treatments induced a significant decrease of ATP, after 24 h-exposures, in H9c2 cells, as shown in Figure 12. The exception was EtOH (EC_{30} and EC_{60}) and Mix C (EC_{30}).

Results on the energetic disturbance were significantly different for COC at EC_{30} (p<0.001, ANOVA/Holm-Sidak's) and for EtOH at EC_{60} (p<0.05, ANOVA/Holm-Sidak's), when compared with results for Mix C, at the same concentrations.

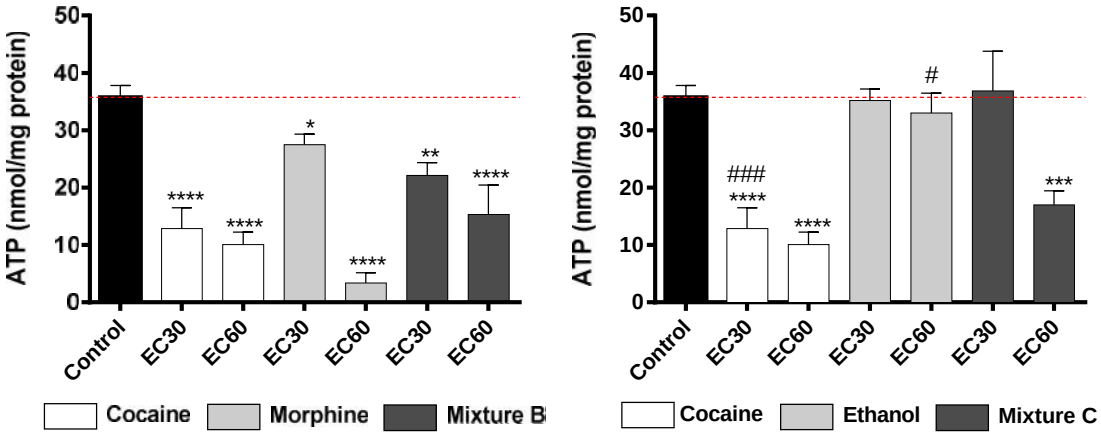

*Figure 12. Effect elicited by cocaine, morphine, ethanol, and mixtures B and C in intracellular ATP, in H9c2 cells, after 24 h-exposure. EC_{30} and EC_{60} are the concentrations producing 30% and 60% of effect, respectively, in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. Mixture B: mixture of COC 1: MOR 2. Mixture C: mixture of COC 1: EtOH 9. Results normalized to the protein content represent the mean ± SEM. Statistical comparisons were made by one-way ANOVA followed by Holm-Sidak's multiple comparison post hoc test. *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001 compared to controls. #p<0.05, ###p<0.001, compared to mixture tested at the same concentration.*

The inability of mitochondria to adequately supply cell with ATP, results in energy deprivation to the cell and potentially necrotic/apoptotic cell death. Microscopic observation of H9c2 cells exposed to our treatments, demonstrated alterations on cell morphology. Compared to controls, these alterations were highly pronounced when cells were exposed to the greatest concentration (EC_{60}; Figure 13).

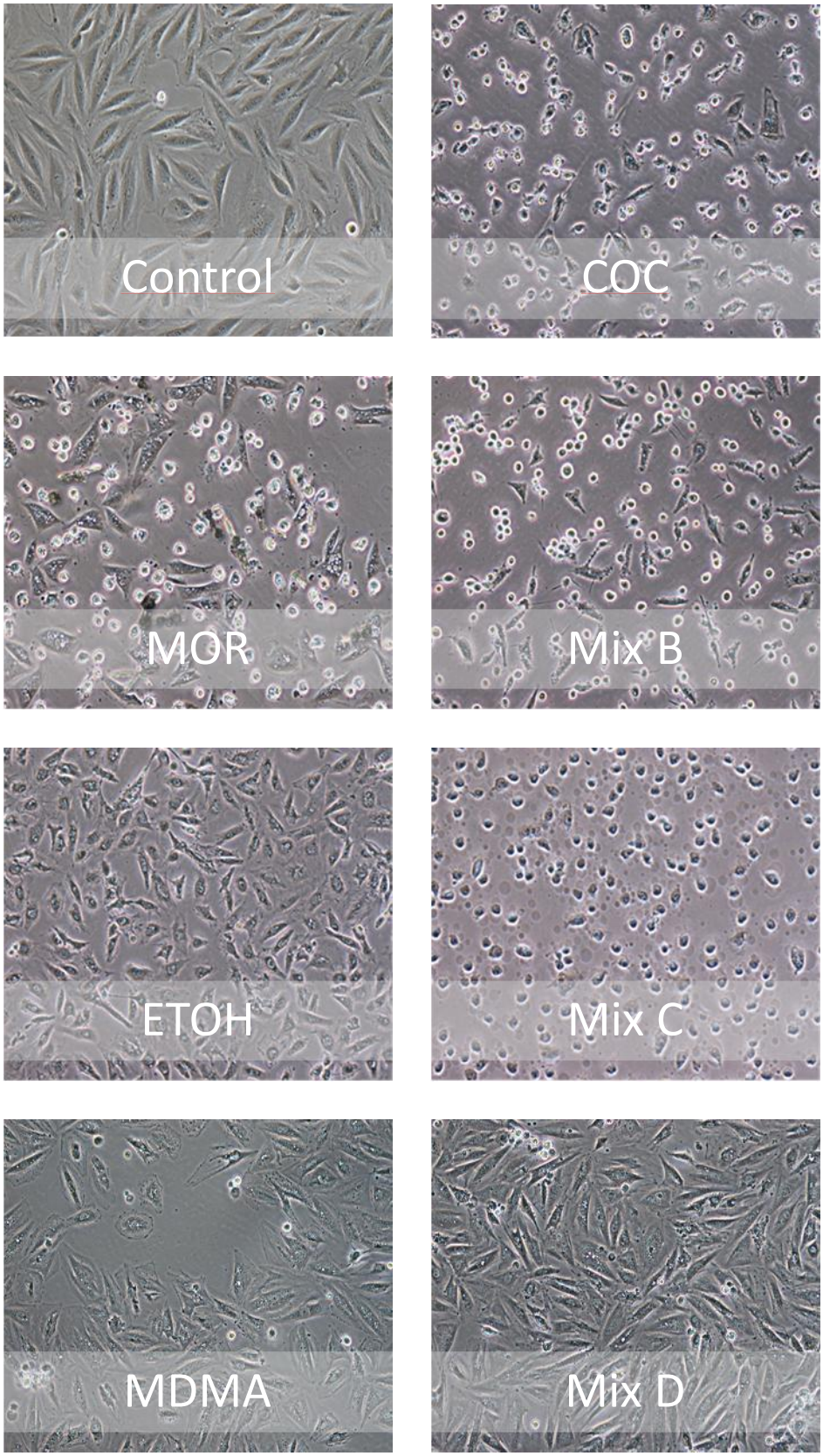

Figure 13. Morphology of H9c2 cells by light microscopy after exposure to cocaine (COC), morphine (MOR), ethanol (EtOH), ecstasy (MDMA), and mixtures B, C, and D, for 24 h at 37°C, at the respective EC_{60}, i.e. the concentration producing 60% of maximal effect in the MTT viability assay. Mix B: mixture of COC 1: MOR 2. Mix C: mixture of COC 1: EtOH 9. Mix D: mixture of COC 1: ecstasy (MDMA) 5. Original magnification ×40.

In an effort to elucidate if these morphological alterations were accompanied by disruption of cytoplasmic membrane integrity, we attempted to perform the evaluation of intracellular LDH leakage. Unfortunately, by the time of the delivery of this dissertation, the conditions of the assay were not yet optimised for our cellular model.

PART V

DISCUSSION

5. Discussion

In the recreational scene, COC is taken along with other licit and illicit drugs of abuse; among them, some of the most frequently associated with the drug are EtOH, HER or its metabolite MOR, and MDMA. Of concern, polydrug abuse is one of the most relevant bewildering factors in establishing the toxicological potential of drugs, as pharmacokinetic and pharmacodynamic interactions may occur (Chan & Anderson, 2014; Collin *et al.*, 2014; Cunha-Oliveira *et al*., 2010; da Silva, Silva, Carvalho, & Carmo, 2014; Farre *et al.*, 1997; Kamendulis *et al.*, 1996; Pontes *et al.*, 2010) and are of serious consequence for the development of unexpected intoxications, which ultimately can be lethal (EMCDDA, 2009; Gossop *et al.*, 2006; Grant & Harford, 1990; Kelly & Parsons, 2008; Leri *et al.*, 2003).

We anticipated that potential interactions between COC and these co-consumed substances could occur, since these drugs present overlapping mechanistic, metabolic and detoxifying pathways (Antolino-Lobo *et al.*, 2011; Chan & Anderson, 2014; He, Brockmoller, Schmidt, Roots, & Kirchheiner, 2008; Pontes *et al.*, 2010; Zhan, Hou, Zhan, & Zheng, 2014). Therefore, we became very interested in evaluating whether COC in combination with EtOH, HER, MOR, or MDMA would be able to yield toxicological effects, distinct from those observed for the single drugs. With this intent, for the toxicological assessment we selected two different *in vitro* models representative of important targets of COC toxicity, *i.e.* liver and heart.

The liver is responsible for the biotransformation and removal of xenobiotics, which implicates their accumulation in the organ prior to metabolic (de)toxification. Accordingly, the principal COC metabolic pathways occur in this organ, among others, mediated by P450 enzymes such as CYP1A, CYP2A, CYP2B, and CYP3A (Yao, Shi, Wang, Gosnell, & Chen, 2013). In addition, the bioactivation of the drug has been pointed out as one of the main causes of its hepatotoxic effects (Boelsterli & Goldlin, 1991; Ndikum-Moffor *et al*., 1998; Pellinen *et al.*, 1994; Thompson, Shuster, & Shaw, 1979).

Heart is further exposed to the formed metabolites through distribution via blood stream, but the organ also contains enzymes whose metabolic activity adds to cardiac toxicity. In accordance, COC is frequently associated with reports of cardiac damage (Bauman *et al.*, 1994; Fan *et al*., 2009; Fineschi *et al.*, 2001; Graziani *et al.*, 2016). Evidence indicates that the drug blocks the reuptake of catecholamines, increasing the release of NA from the adrenergic nerve terminals; the activation of sympathomimetic pathways results in augmentation of ventricular contractility, increased blood pressure, heart rate, and myocardial oxygen demand (Bauman *et al.*, 1994; O'Leary & Hancox, 2010). Additionally, the blockage of the voltage-gated sodium channels has also been implicated in COC-induced heart damage (Afonso *et al.*, 2007; Awtry & Philippides, 2010; Hollander, 2008).

Although cardiotoxicity of COC have traditionally been attributed to sympathomimetic stimulation, compelling evidence proved that COC and its metabolites may exert direct toxicity (Valente *et al*., 2012; Welder, Smith, Ramos, & Acosta, 1988; Xu, Flick, Mitchel, Knowles, & Ault, 1999).

So, in the current work we chose to evaluate the toxicity of a few mixtures of COC in two *in vitro* models for the hepatocyte and cardiomyocyte, namely primary hepatocytes isolated from rat liver and immortalized H9c2 rat cells, respectively.

H9c2 cells have been widely used in toxicity studies as a useful *in vitro* model for the cardiomyocyte (Arbo *et al*., 2014; Begieneman *et al.*, 2016; Sardao, Oliveira, Holy, Oliveira, & Wallace, 2007). These cells display morphological features similar to those of immature embryonic cardiomyocytes but maintain several biochemical characteristics of adult cardiomyocytes (Hescheler *et al.*, 1991); they form confluent multinuclear cells (myotubes) and present electrical and signalling mechanisms characteristic of cardiac cell (Kimes & Brandt, 1976). The L-type voltage-dependent calcium channels and the pattern of signal-transducing G proteins of H9c2 cells are cardiac-specific. They are rich in rough endoplasmic reticulum, the cell surface is often enlarged by microvilli, and its sugar residues coating is similar to that found in isolated rat cardiomyocytes (Hescheler *et al.*, 1991). These cells express genes encoding for cardiac sarcomeric proteins, such as troponin T, calcium transporters and associated machinery (Branco *et al.*, 2015).

In what concerns our choice regarding the hepatic model, primary hepatocytes remain the *gold standard* for liver *in vitro* toxicity studies. Compared to the established cell lines, primary hepatocytes are more representative of the *in vivo* situation, as they possess the same phenotypic and genotypic characteristics. Contrary, most currently used liver-derived immortalized cells, such as HepG2, are derived from hepatic tumours and display an altered expression profile of liver-specific functions (Gerets *et al.*, 2012). Due to the high proliferative capacity of these cells, the expression profile of many liver-specific genes has been shown to vary between passages and, consequently, data from immortalized hepatocytes may lack reproducibility and consistency across laboratories or among experiments set in different occasions. Still, considering the drawbacks of using primary cells (limited life span, low throughput, animal ethical concerns, costs associated to animal maintenance and care, inter-individual variability and, consequently, low reproducibility), we pondered for our study the use of a recently developed human hepatoma cell line, HepaRG cells, as it retains high expression of many liver-specific functions, such as cytochrome P450s, nuclear receptors, transcription, membrane transporters, and phase II enzymes (Gerets *et al.*, 2012). Unfortunately, these cells were established from a donor who was CYP2E1 and CYP2D6 poor metabolizer, and these CYP450 isoforms are very important for the metabolism of our testing drugs (Carmo *et al.*, 2006 ; Koop & Tierney, 1990).

Our results from H9c2 cardiomyocyte exposures show that MDMA (EC_{50} 1.07 mM) was the most toxic drug. Previously, Carvalho *et al* (2004) observed no toxicity when freshly isolated adult rat cardiomyocytes were exposed to MDMA for 4 h, at concentrations up to 1.6 mM. In the same work, these authors proved that MDMA metabolic activation was required for the manifestation of MDMA *in vitro* cardiotoxicity. It is possible that in our work the longer incubation with the drug could compensate the relatively low metabolic competence of the heart, as toxicity was expressed at lower concentrations than those tested by Carvalho *et al.* This hypothesis is supported by the higher detrimental effects observed for MDMA in primary hepatocytes (EC_{50} 0.56 mM) – higher metabolic rates are admitted for this cellular model. The hepatotoxicity observed herein for MDMA was slightly lower than that previously reported (EC_{50} 1.070 mM) at similar experimental settings (Valente *et al*., 2016).

Contrarily, EtOH was the least potent drug in inducing cell death, both in H9c2 (EC_{50} 305.26 mM) and in primary hepatocytes (EC_{50} 263.26 mM). Some discrepancies exist regarding former reported toxicities; one study describes that 50 mM EtOH reduced viability of cultured primary rat hepatocytes to ~80%, only after 5h-incubation (Collin *et al.*, 2014), while another one performed in cultured human hepatocytes more closely resemble our data (EC_{50} 330 mM; LDH assay after 24 h-incubation). Other studies on the mode of cell death, showed that EtOH concentrations up to 100 mM induced apoptosis in a concentration-dependent manner, while from 200 mM on cardiomyocytes isolated from Sprague-Dawley rats preferentially died by necrosis, when treated for 24 h (Guan, Lui, Morkin, & Bahl, 2004). These hepatic and cardiac effects seem to be mediated by tumour necrosis factor-alpha (TNF-α) since increased levels of TNF-α receptor-1 (TNF-R1) were observed after 24 h-exposure to EtOH, both in rat hepatoma cells (90% at 50 mM and 230% at 100 mM) and in neonatal rat primary cardiomyocytes (36% at 50 mM and 44% at 100 mM).

Also COC was ~2.4 times less detrimental for H9c2 cells (EC_{50} 2.60 mM), when compared to primary hepatocytes (EC_{50} 1.07 mM). Few studies offered evidence that *in vitro* cell death elicited by COC significantly varies across distinct cellular models and experimental settings (Jover, Ponsoda, Gomez-Lechon, & Castell, 1993; LeDuc *et al.*, 1994; Zaragoza, Diez-Fernandez, Alvarez, Andres, & Cascales, 2001). For instance, slight LDH leakage was observed when neonatal Sprague-Dawley rat myocardial cells where exposed to 1 mM COC for 24 h (Yuan & Acosta, 2000), but that concentration was close to the EC_{50} observed for hepatocytes isolated from male Sprague-Dawley (EC_{50} 1.10 mM), which is in line with our findings (Jover et al., 1993).

Compared to the other drugs, the lower cardiotoxicity of MORPH (EC_{50} 6.93 mM) may be related to the H9c2 protective effects that were described for this drug, when present at low concentrations (Amini-Khoei et al., 2016).

Curiously, in what concerns HER hepatotoxicity (EC_{50} 0.26 mM), our data do not reproduce results from other assays in cultured human hepatocytes, in which toxicity after 24 h incubation was only achieved at higher concentrations (EC_{50} 3.6 mM for LHD assay; EC_{50} 3.9 mM for MTT assay) (Jover et al., 1993). Although some interspecies variability might be at play, in addition to the fact that extremely different cell culture conditions were used by the authors of this study, we acknowledge that these differences are very significant.

In general, with exception of MORPH and HER, which were only tested in one cellular model and therefore comparisons are precluded, all tested drugs disclosed higher toxicity in primary hepatocytes. These data suggest that the functional specificity of the organ from which cells derive might be implicated in the toxicological discrepancies observed. Of particular interest, metabolism may present relevant implications for the expression of toxicity in hepatocytes where the levels of enzymes are more preponderant; even in the absence of metabolic activation (such as the cases of MDMA, COC, and EtOH, whose metabolites are more detrimental than the parent drugs), the metabolic pathways involved in the detoxification processes are great generators of ROS/RNS (e.g. NADPH oxidase activity). In the same way, drugs that more extensively affect calcium signalling pathways will probably impact cardiomyocyte more severely; for example, COC inhibits inwardly rectifying potassium channels activated by G protein (GIRK, Kir3), impairing regulation of cardiomyocyte excitability and contractility; EtOH has the opposite action (Kobayashi, Nishizawa, & Ikeda, 2011).

In addition, the differential expression of CYP isoforms across distinct species can be responsible for differences in susceptibility to the drug toxicity (Pellinen *et al*., 2000). For instance, in mouse, CYP2A, 2B, and 3A have been shown to catalyse COC N-demethylation (Aoki, Takimoto, Ota, & Yoshida, 2000; Bornheim, 1998; Pellinen et al., 1994; Pellinen *et al.*, 1996). This reaction is catalysed by CYP3A and CYP2B in rat, and by CYP3A in humans (Boelsterli, Lanzotti, Goldlin, & Oertle, 1992; LeDuc *et al.*, 1993; A. P. Li, Kaminski, & Rasmussen, 1995; Pellinen *et al.*, 1994; Poet, Brendel, & Halpert, 1994; Poet, McQueen, & Halpert, 1996). These interspecies metabolic differences were connected to differences observed in liver damage. Comparing the three species, mouse and human are the most and the least sensible specie to COC hepatotoxicity, respectively (Connors *et al.*, 1990).

Owing information on toxicity for the single drugs, we were then able to proceed to the mixture assessments. One of our main motivations for these mixture studies was to

address the concern that toxicological profiling of single drugs might grossly underestimate combined toxicity due to the potential for occurrence of synergism or additivity between mixture components, such has been observed for other drugs of abuse (*e.g.* amphetamine- and piperazine-derivatives) (Antia, Tingle, & Russell, 2009a, 2009b; da Silva *et al.*, 2014; Dias da Silva *et al.*, 2016).

The assessment of the combination effects in terms of synergy, antagonism and additivity, relies on the accurate prediction of the expected mixture effects. When we started our investigations, most of the studies conducted with COC and their associations, including those claiming synergisms {Duvauchelle, 1998 #1196; Aberg, 2007 #1189; Sobel, 1999 #1122; Cunha-Oliveira, 2010 #1170; Czoty, 2015 #1124; Daza-Losada, 2008 #1177; {Diller, 2007 #1187} were designed with no reference to the expected joint effects, nor to the conceptual principles, such as the CA and IA, which are the foundation of mixture additivity. Also, all too often, mixture expectations were inaccurately set, for example by admitting *'effect summation'* (Duvauchelle, Sapoznik, & Kornetsky, 1998).

In what concerns the prediction models used herein, it is generally accepted that CA produces accurate estimations of the effects of mixtures containing drugs that act by similar mechanisms; therefore, each individual drug will contribute to the overall mixture effect in proportion to its concentration. So, it is expectable that when one mixture component is replaced by an equieffective concentration of another one, the same overall effect will be observed. Contrarily, the IA model produces more robust predictions for mixtures of drugs that have distinct mechanisms of action; also, it has been successfully applied to mixtures of drugs whose mechanisms are unknown (Cedergreen *et al.*, 2008). Notwithstanding, it often happens that CA and IA generate very identical expectations of additive mixture effects, which cannot be distinguished from one another. Examples of this problem were found when we tested our binary mixtures of COC with commonly consumed drugs. Accordingly, the effect predictions derived from CA and IA for the six mixtures tested were not sufficiently different to enable choosing the most accurate model for prediction of mixture effects – although CA demonstrated to be a model slightly more conservative and, therefore, more protective, as its EC_{50} values were marginally smaller than those achieved for IA.

It has been shown that the discrimination between predictions derived from CA and IA relies on the number of the components of the mixture, their fraction in the mixture, and on the slopes of their concentration-response fits (Drescher & Boedeker, 1995). Thus, in our study, in order to validate the applicability of these models, one of the following approaches would have to be considered: i) selection of a different regression model for describing the relationships concentration *versus* effects of the single agents, or changing the *in vitro* system used (*e.g.* other cellular model) in order to alter the slope of the individual

drugs; ii) changing the mixture ratios; or iii) increasing the number of mixture components. Even tough, none of these strategies would assure clear distinction between CA and IA. In addition, all settings of our experiments were meticulously selected; for instance, the choice of the nonlinear regressions to fit experimental data was based on *best fit* statistical criteria, the combination ratios were based on mixtures that occurred in realistic situations, and by using binary mixtures we intended to reduce the complexity and the confounding factors associated with the toxicological assessment of mixtures.

Regarding the deviations from additivity reported in this work, experimental effects obtained for Mix A in primary rat hepatocytes were shifted to left, compared to the computed expectations, and are therefore consistent with antagonism. Previous studies demonstrated that in humans the hydrolyses of HER to 6-MAM, and of 6-MAM to morphine by liver PChE and carboxylesterases hCE1 and hCE2 are competitively inhibited by COC (Kamendulis *et al*., 1996). Since the prediction models that we used to assess mixture toxicity do not take into account pharmacokinetic or pharmacodynamic interactions, and the metabolism of these two drugs is catalysed by common enzymatic pathways in liver, it is plausible that the occurrence of metabolic competition could result in the antagonism here reported, since COC metabolites were demonstrated to be highly toxic (Boelsterli & Goldlin, 1991; Valente *et al.*, 2012).

Contrary to Mix A, a synergism was verified for Mix C and it may also be explained by the occurrence of metabolic interactions. Accordingly, studies in humans showed that plasma concentrations of benzoylecgonine decrease in the drug combination settings, while COC concentrations increase (Cami, Farre, Gonzalez, Segura, & de la Torre, 1998). This strongly supports the hypothesis of liver carboxylesterases inhibition; COC is metabolized to benzoylecgonine by the action of liver hCE1; in the presence of EtOH, the carboxylesterase catalyses the ethyl transesterification of COC into CE; of note, the active metabolites CE and norcocaethylene were only observed in the mixture settings (Cami *et al.*, 1998) and CE has lower binding affinity to the esterase, leading to a longer elimination half-life (Brzezinski *et al.*, 1997). On the other hand, the pathway of NCOC formation through CYP 3A4 seems to be privileged in the presence of EtOH, as plasma concentrations of NCOC were higher for the mixture administration (Cami *et al.*, 1998). As mentioned, NCOC is metabolized to the free radical NCOC-NO$^{\cdot}$ (Pellinen *et al.*, 2000), which induces intense oxidative stress and ultimately impairs the hepatocellular function (Charkoudian & Shuster, 1985). Consequentially, the differential profile of metabolites that arise from the combination may severely alter the overall hepatotoxicity, when these drugs co-occur.

For Mix D, the effects observed in hepatocytes seem to be dependent on the mixture concentration; at low concentrations, MDMA and COC seem to interact antagonistically; the opposite occurs above the EC_{50}, when lower concentrations than predicted were required

to produce the same effect (*i.e.* synergism). Interestingly, a similar complex concentration-dependent pattern of mixture effects had been previously observed, when Diller and colleagues (2007) measured behavioural and neurochemical effects of MDMA and COC in combination. These authors reported that increasing doses of COC resulted in antagonistic CPP effects, when MDMA was at lower doses; but this pattern of antagonism was reversed at higher doses of MDMA. In this report, pharmacodynamic interactions might be underlying mixture effects, since COC and MDMA trigger similar dopaminergic mechanisms (Rothman & Baumann, 2003). In our study, however, pharmacokinetic interactions are more feasible; COC inhibits CYP2D6 (Shen *et al*., 2007), the CYP450 isoform that catalyses ~30% MDMA metabolism (Schmid *et al.*, 2016) and whose polymorphism may severely impact the expression of MDMA toxicity (Carmo *et al.*, 2006). Another noticeable aspect of Mix D results is the steeper slope of the experimental curve, compared to the slope of the CA and IA predictions. Concentration-mortality curves presenting such slopes are of utmost toxicological relevance, as it indicates that very slight increases in the concentration of the mixture promote significant increments in cytotoxicity, which may quickly shift from roughly undetectable to maximal mortality (Dias da Silva, Silva, & Carmo, 2013).

With the exception of the combination with EtOH (Mix C), the toxicity of COC binary mixtures was potentiated in H9c2 cells, as the drug acted synergistically both with MDMA (Mix D) and MOR (Mix B). Duvauchelle *et al* (1998) advocated the synergic reinforcing effects of COC and MOR diacetate combinations when they observed higher number of reinforcements for the combination, compared with each drug alone. Although we acknowledge the relevance of the reported effects, caution should be taken by the authors when declaring synergisms; in the work cited no reference to the expected effects was done and, since the dose-response curves did not follow a linear pattern, the '*effect summation'* concept is not applied. As far as we concerned, the observed effects may also be consistent with antagonism or additivity. Chemical interactions between these drugs, such as COC-MOR adduct formation have been strongly debated (Cunha-Oliveira *et al.*, 2010; Garrido *et al.*, 2007). COC-MOR adducts impair the interaction of the single drugs with the respective molecular targets (*e.g.* efflux/ influx transporters, receptors, enzymes, etc.) and may trigger different signalling pathways. Compared to liver, such adducts are more likely to be produced in heart, were the drugs are less metabolised or at a slower rate. These mechanisms of adduct formation may contribute for the interaction that we report herein for Mix B. In addition, Cunha-Oliveira *et al*. (2010) demonstrated that when COC and HER (rapidly converted to MOR) are co-exposed they elicit more neurotoxicity than each drug alone and the mode of co-exposure [i.e. sequential (HER followed by COC) or simultaneous (HER: COC)] changed the cell death pathways triggered by the single drugs; in case of

HER:COC, cell death mechanisms were shifted towards necrosis, which is extremely more harmful than apoptosis.

The synergism observed for Mix D might be a result of the overlapping mechanisms of cardiac injury triggered by both COC and MDMA, which include induction of oxidative stress (Graziani *et al.*, 2016; Song, Moon, Upreti, Eddington, & Lee, 2010) with consequent modulation of redox-sensitive signalling pathways, such as mitogen-activated protein kinase (MAPK) superfamily (Fan *et al*., 2009; Koczor *et al.*, 2015). In agreement, COC activates MAPKs, in particular extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, and c-Jun NH2-terminal kinase phosphorylation, through increased Nox2 oxidase activity (Fan *et al*., 2009). Activation of ERK / PI3K/ Akt signal transduction pathways were also observed following MDMA exposure (Goni-Allo *et al*., 2008; Koczor *et al.*, 2015). In addition, recent evidence also involved the modulation of ATP-sensitive potassium (K_{ATP}) channels in the toxicity expressed MDMA and COC (Goni-Allo *et al*., 2008; Reyes, Kane, Zingman, Yamada, & Terzic, 2009).

For Mix C, the effects experimentally obtained were in agreement with values estimated by CA and IA models (additivity). Similarly, when isolated cardiomyocytes were exposed to COC and ETOH concomitantly, significant greater inhibitory effects on contractility and on calcium transient amplitude were observed, compared to either drug alone, and these results were consistent with the additive predictions calculated assuming that drugs acted independently at distinct targets within the excitation-contraction coupling pathway (Nicolas, Rubin, & Thomas, 1996). In spite of the existence of other reports of additivity between COC and EtOH, in our opinion these studies provided no evidence of such effects (Uszenski, Gillis, Schaer, Analouei, & Kuhn, 1992).

Also, although metabolic interactions have been mostly highlighted as the reason for experimental deviations from our additivity expectations, particularly in hepatocyte, other causes should be considered, such as the competition at other drug targets (*e.g.* transcription factors, P-glycoprotein efflux pump, membrane receptors, etc.), fraction bound to serum proteins or retained in the lipid bilayer membrane, which might decrease as it is displaced from the binding sites by the remaining mixture components, differences concerning immortalised *versus* primary cells, etc.

As depicted through this dissertation, the formation of reactive species is one of the most prominent toxicity mechanisms for the drugs tested herein (Costa, Carvalho, Duarte, Bastos Mde, & Remiao, 2013). Accordingly, our experimental results demonstrated a concentrations-dependent increase in ROS/RNS for all drugs (COC, MOR, MDMA and EtOH), individually or combined. In accordance, oxidative stress was linked to cardiomyopathy induced by EtOH, through the accumulation of acetaldehyde, which is

highly reactive and attacks several vital cell structures, such as mitochondria (Guo & Ren, 2010 ; Niemela, 2007; Vendemiale *et al.*, 2001).

In heart, the production of reactive species by modulators of serotonergic and dopaminergic functions (such as COC and MDMA) is generally explained by the elevation of catecholamines and, subsequently, either by the stimulation of adrenoreceptors, or by the enzymatic/ non enzymatic degradation of catecholamines. In our in vitro system, however, cultured cardiomyocytes are devoid of enervation, hormonal control, and influence of local biological factors, which improves the mechanistic investigation of direct drug-induced cardiotoxicity; thus, the contribution of these mechanisms for COC cardiotoxicity were here abolished and the oxidative stress generated only attributed to the direct cardiotoxic actions of these drugs (COC, MDMA, MOR, and EtOH).

Nevertheless, significant oxidative stress was only evident at the highest concentration tested (EC60, $p<0.0001$), suggesting that oxidative phenomena might be counteracted by the intrinsic machinery of cellular defence, at lower concentrations; for example, GSH is a cytosolic redox buffer which displays a pivotal role in cell defence against redox injury (Griffith, 1999). Binary mixtures of COC with MOR or with EtOH, and the respective single drugs, decrease cytosolic GSH and, concurrently, promoted a slight increase of GSSG. These observations are corroborated by studies in rat heart after chronic COC administration (Fineschi *et al.*, 2001). In vitro experimental data demonstrated that incubation of isolated adult rat cardiomyocytes with MDMA metabolites decreased GSH levels and antioxidant enzyme activities, with loss of normal cell morphology (Carvalho *et al.*, 2004); and Shenouda *et al*. (2008) observed an increase in nitrated tyrosine residues, which is as a marker of oxidative stress. However, in our study GSH/GSSG levels were not evaluated for MDMA nor Mix D.

As a high cytosolic GSH/GSSG ratio is important for cellular antioxidant homeostasis (Neri *et al.*, 2007), it is conceivable that GSSG is being extruded to the extracellular environment. On the other hand, the discrepant high depletion of GSH, compared to the slight GSSG increase, may occur by formation of adducts, such was already reported for metabolites of MDMA, COC and EtOH (Blair, 2006; Cole & Deeley, 2006; Hiramatsu, Kumagai, Unger, & Cho, 1990; Sultana, Bhupanapadu Sunkesula, Sharma, Reddanna, & Babu, 2005).

The excessive generation of reactive species promotes an irreversible modification of lipids and proteins, compromising the function of vital cell structures such as enzymes, membranes and transporters. In vivo studies with COC demonstrated the increase of malondialdehyde (indicative of oxidative damage to cell membranes), GSSG, and protein carbonyls; and decreased of GSH, manganese superoxide dismutase (MnSOD) responsible for removing the superoxide anion radical and converting it into hydrogen

peroxide (H_2O_2), glutathione peroxidase (GPX) which convert this by-product into water, catalase, and glutathione-S-transferase (GST) (Costa *et al.*, 2011 ; Neri *et al.*, 2007). In in vivo studies conducted in adult mice treated with EtOH for six hours, myocardial ultrastructure alterations, possible caused by myocardial lipid peroxidation and protein oxidation, were also observed (Kannan, Wang, & Kang, 2004).

The cellular structure and functional integrity may be compromised by reactive species when cell defence capability is surpassed, as these entities will interact with critical biologic molecules, such as lipids, nucleic acids, and proteins, promoting the oxidative degradation. Also, excessive formation of reactive species can affect the production of ATP by impairing oxidative phosphorylation in mitochondrial electron transport through deregulation of Ca^{2+} sequestration in the matrix mitochondrial and disruption of $\Delta\psi m$ (GM, 2000). Our experimental results are in line with these mechanisms as mitochondrial membrane hyperpolarization and a significant decline of ATP levels were observed. Mitochondrial injury by its turn increases the membrane permeability of the organelle with consequent release of pro-apoptotic factors. Besides impacting cellular metabolic functions, ATP levels decide the fate of the cell towards necrosis or apoptosis (energy-dependent process), corroborating our observations of the altered cell morphology. In vitro studies demonstrated that cardiomyocytes exposed to EtOH for 24 h died by apoptosis at lower doses, and by necrosis at higher; these effects were mediated by intracellular increase of ROS and decrease of mitochondrial membrane potential (S. Y. Li *et al.*, 2006).

PART VI

CONCLUSION

6. Conclusion

The toxicological assessment of mixtures is generally obstructed by the overwhelming amount of conceivable combinations to which individuals might be exposed to. Coherently, the use of predictive models to calculate the effects of combinations of chemicals, using information on toxicity of the individual drugs, would be of relevant application. Herein, we applied for the first time the concepts of CA and IA to evaluate the joint effects of COC and a number of frequently co-consumed substances – HER/MOR, MDMA, and EtOH – that are known to be concomitantly ingested, in corresponding recreational scene. Of concern, the toxicological impact of the co-occurrence of COC and these substances could hardly be anticipated by these two broadly employed models for the calculation of mixture effects of chemicals. Our predictions according to these principles, which are widely used for estimating toxicity of chemical mixtures in several fields, proved to be inadequate for the appraisal of the joint effects of mixtures of COC. This conclusion hold true when several binary mixtures of the drug were tested at realist drug ratios, in two different in vitro systems that are representative of major target organs for the toxicity of this drug, i.e. liver and heart. All observed deviations from additivity probably indicate that pharmacokinetic interactions are at play.

Notably, several additive effects that conformed to CA and synergisms were observed, denoting that the co-occurrence of other drugs greatly altered COC individual toxicity. Both manifestations are worrisome and, in a clinical perspective, may imply important toxicological consequences for the consumer. Overall, our results emphasize the risks at which consumers of these drugs are exposed to, when engaging in polydrug abuse practices.

Evidence from our work also indicates that the effects of a particular mixture might be significantly different among the distinct target organs, suggesting that, depending on their functional specificity, differential mechanisms of toxicity or protection might be at play. As hepatocytes revealed greater susceptibility to individual and combined drug-induced toxicity, compared to H9c2 cardiomyocytes, and admitting that this model is metabolically more competent, this might indicate that cells are more vulnerable when exposed to substances that are metabolised into harmful metabolites.

The mechanistic evaluation on the pathways triggered by these drugs and their mixtures revealed that increase oxidative stress with concurrent decline of antioxidant defences, dramatic depletion of intracellular ATP, and hyperpolarization of mitochondria might be strongly correlated to the observed cardiomyocyte toxicity.

PART VII

REFERENCES

7. References

Aberg, M., Wade, D., Wall, E., & Izenwasser, S. (2007). Effect of MDMA (ecstasy) on activity and cocaine conditioned place preference in adult and adolescent rats. Neurotoxicol Teratol, 29(1), 37-46.

Achat-Mendes, C., Anderson, K. L., & Itzhak, Y. (2003). Methylphenidate and MDMA adolescent exposure in mice: long-lasting consequences on cocaine-induced reward and psychomotor stimulation in adulthood. Neuropharmacology, 45(1), 106-115.

Afonso, L., Mohammad, T., & Thatai, D. (2007). Crack whips the heart: a review of the cardiovascular toxicity of cocaine. Am J Cardiol, 100(6), 1040-1043.

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., & Grimme, L. H. (2000). Predictability of the toxicity of multiple chemical mixtures to Vibrio fischeri: Mixtures composed of similarly acting chemicals. Environmental Toxicology and Chemistry, 19(9), 2341-2347.

Amini-Khoei, H., Hosseini, M. J., Momeny, M., Rahimi-Balaei, M., Amiri, S., Haj-Mirzaian, A., . . . Dehpour, A. R. (2016). Morphine Attenuated the Cytotoxicity Induced by Arsenic Trioxide in H9c2 Cardiomyocytes. Biol Trace Elem Res, 173(1), 132-139.

Andersson, M., Bjorkhem-Bergman, L., & Beck, O. (2015). Possible mechanism for inhibition of morphine formation from 6-acetylmorphine after intake of street heroin. Forensic Sci Int, 252, 150-156.

Antia, U., Tingle, M. D., & Russell, B. R. (2009a). In vivo interactions between BZP and TFMPP (party pill drugs). N Z Med J, 122(1303), 29-38.

Antia, U., Tingle, M. D., & Russell, B. R. (2009b). Metabolic interactions with piperazine-based 'party pill' drugs. J Pharm Pharmacol, 61(7), 877-882.

Antolino-Lobo, I., Meulenbelt, J., Nijmeijer, S. M., Maas-Bakker, R. F., Meijerman, I., van den Berg, M., & van Duursen, M. B. (2011). 3,4-methylenedioxymethamphetamine (MDMA) interacts with therapeutic drugs on CYP3A by inhibition of pregnane X receptor (PXR) activation and catalytic enzyme inhibition. Toxicol Lett, 203(1), 82-91.

Aoki, K., Takimoto, M., Ota, H., & Yoshida, T. (2000). Participation of CYP2A in cocaine-induced hepatotoxicity in female mice. Pharmacol Toxicol, 87(1), 26-32.

Arbo, M. D., Silva, R., Barbosa, D. J., da Silva, D. D., Rossato, L. G., Bastos Mde, L., & Carmo, H. (2014). Piperazine designer drugs induce toxicity in cardiomyoblast h9c2 cells through mitochondrial impairment. Toxicol Lett, 229(1), 178-189.

Aslibekyan, S., Levitan, E. B., & Mittleman, M. A. (2008). Prevalent cocaine use and myocardial infarction. Am J Cardiol, 102(8), 966-969.

Awtry, E. H., & Philippides, G. J. (2010). Alcoholic and cocaine-associated cardiomyopathies. Prog Cardiovasc Dis, 52(4), 289-299.

Bailey, D. N. (1994). Studies of cocaethylene (ethylcocaine) formation by human tissues in vitro. J Anal Toxicol, 18(1), 13-15.

Bauman, J. L., Grawe, J. J., Winecoff, A. P., & Hariman, R. J. (1994). Cocaine-related sudden cardiac death: a hypothesis correlating basic science and clinical observations. J Clin Pharmacol, 34(9), 902-911.

Begieneman, M. P., Ter Horst, E. N., Rijvers, L., Meinster, E., Leen, R., Pankras, J., . . . Krijnen, P. A. (2016). Dopamine induces lipid accumulation, NADPH oxidase-related oxidative stress and a pro-inflammatory status of the plasma membrane in H9C2 cells. Am J Physiol Heart Circ Physiol

Blair, I. A. (2006). Endogenous glutathione adducts. Curr Drug Metab, 7(8), 853-872.

Boelsterli, U. A., & Goldlin, C. (1991). Biomechanisms of cocaine-induced hepatocyte injury mediated by the formation of reactive metabolites. Arch Toxicol, 65(5), 351-360.

Boelsterli, U. A., Lanzotti, A., Goldlin, C., & Oertle, M. (1992). Identification of cytochrome P-450IIB1 as a cocaine-bioactivating isoform in rat hepatic microsomes and in cultured rat hepatocytes. Drug Metab Dispos, 20(1), 96-101.

Bornheim, L. M. (1998). Effect of cytochrome P450 inducers on cocaine-mediated hepatotoxicity. Toxicol Appl Pharmacol, 150(1), 158-165.

Bortolotti, F., Gottardo, R., Pascali, J., & Tagliaro, F. (2012). Toxicokinetics of cocaine and metabolites: the forensic toxicological approach. Curr Med Chem, 19(33), 5658-5663.

Branco, A. F., Pereira, S. P., Gonzalez, S., Gusev, O., Rizvanov, A. A., & Oliveira, P. J. (2015). Gene Expression Profiling of H9c2 Myoblast Differentiation towards a Cardiac-Like Phenotype. PLoS One, 10(6), e0129303.

Brown, E., Prager, J., Lee, H. Y., & Ramsey, R. G. (1992). CNS complications of cocaine abuse: prevalence, pathophysiology, and neuroradiology. AJR Am J Roentgenol, 159(1), 137-147.

Brzezinski, M. R., Spink, B. J., Dean, R. A., Berkman, C. E., Cashman, J. R., & Bosron, W. F. (1997). Human liver carboxylesterase hCE-1: binding specificity for cocaine, heroin, and their metabolites and analogs. Drug Metab Dispos, 25(9), 1089-1096.

Caine, S. B., Negus, S. S., Mello, N. K., Patel, S., Bristow, L., Kulagowski, J., Borrelli, E. (2002). Role of dopamine D2-like receptors in cocaine self-administration: studies with D2 receptor mutant mice and novel D2 receptor antagonists. J Neurosci, 22(7), 2977-2988.

Cami, J., Farre, M., Gonzalez, M. L., Segura, J., & de la Torre, R. (1998). Cocaine metabolism in humans after use of alcohol. Clinical and research implications. Recent Dev Alcohol, 14, 437-455.

Caplan, Y. H. (2015). Disposition of toxic drugs and chemicals in man. J Anal Toxicol, 39(5), 417.

Carmo, H., Brulport, M., Hermes, M., Oesch, F., Silva, R., Ferreira, L. M., . . . Hengstler, J. G. (2006). Influence of CYP2D6 polymorphism on 3,4-methylenedioxymethamphetamine ('Ecstasy') cytotoxicity. Pharmacogenet Genomics, 16(11), 789-799.

Carroll, F. I., Gao, Y., Abraham, P., Lewin, A. H., Lew, R., Patel, A., . . . Kuhar, M. J. (1992). Probes for the cocaine receptor. Potentially irreversible ligands for the dopamine transporter. J Med Chem, 35(10), 1813-1817.

Carvalho, M., Remiao, F., Milhazes, N., Borges, F., Fernandes, E., Monteiro Mdo, C., Bastos, M. L. (2004). Metabolism is required for the expression of ecstasy-induced cardiotoxicity in vitro. Chem Res Toxicol, 17(5), 623-632.

Cedergreen, N., Christensen, A. M., Kamper, A., Kudsk, P., Mathiassen, S. K., Streibig, J. C., & Sorensen, H. (2008). A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. Environ Toxicol Chem, 27(7), 1621-1632.

Chan, L. N., & Anderson, G. D. (2014). Pharmacokinetic and pharmacodynamic drug interactions with ethanol (alcohol). Clin Pharmacokinet, 53(12), 1115-1136.

Charkoudian, J. C., & Shuster, L. (1985). Electrochemistry of norcocaine nitroxide and related compounds: implications for cocaine hepatotoxicity. Biochem Biophys Res Commun, 130(3), 1044-1051.

Cole, S. P., & Deeley, R. G. (2006). Transport of glutathione and glutathione conjugates by MRP1. Trends Pharmacol Sci, 27(8), 438-446.

Collin, A., Hardonniere, K., Chevanne, M., Vuillemin, J., Podechard, N., Burel, A., Sergent, O. (2014). Cooperative interaction of benzo[a]pyrene and ethanol on plasma membrane remodeling is responsible for enhanced oxidative stress and cell death in primary rat hepatocytes. Free Radic Biol Med, 72, 11-22.

Cone, E. J., Hillsgrove, M., & Darwin, W. D. (1994). Simultaneous measurement of cocaine, cocaethylene, their metabolites, and "crack" pyrolysis products by gas chromatography-mass spectrometry. Clin Chem, 40(7 Pt 1), 1299-1305.

Connors, S., Rankin, D. R., Gandolfi, A. J., Krumdieck, C. L., Koep, L. J., & Brendel, K. (1990). Cocaine hepatotoxicity in cultured liver slices: a species comparison. Toxicology, 61(2), 171-183.

Costa, V. M., Carvalho, F., Bastos, M. L., Carvalho, R. A., Carvalho, M., & Remiao, F. (2011). Contribution of catecholamine reactive intermediates and oxidative stress to the pathologic features of heart diseases. Curr Med Chem, 18(15), 2272-2314.

Costa, V. M., Carvalho, F., Duarte, J. A., Bastos Mde, L., & Remiao, F. (2013). The heart as a target for xenobiotic toxicity: the cardiac susceptibility to oxidative stress. Chem Res Toxicol, 26(9), 1285-1311.

Crespi, D., Mennini, T., & Gobbi, M. (1997). Carrier-dependent and Ca(2+)-dependent 5-HT and dopamine release induced by (+)-amphetamine, 3,4-methylendioxymethamphetamine, p-chloroamphetamine and (+)-fenfluramine. Br J Pharmacol, 121(8), 1735-1743.

Cunha-Oliveira, T., Rego, A. C., Garrido, J., Borges, F., Macedo, T., & Oliveira, C. R. (2010). Neurotoxicity of heroin-cocaine combinations in rat cortical neurons. Toxicology, 276(1), 11-17.

Czoty, P. W. (2015). Effects of chronic binge-like ethanol consumption on cocaine self-administration in rhesus monkeys. Drug Alcohol Depend.

da Silva, D. D., Silva, E., Carvalho, F., & Carmo, H. (2014). Mixtures of 3,4-methylenedioxymethamphetamine (ecstasy) and its major human metabolites act additively to induce significant toxicity to liver cells when combined at low, non-cytotoxic concentrations. J Appl Toxicol, 34(6), 618-627.

Dackis, C. A., & Gold, M. S. (1985). New concepts in cocaine addiction: the dopamine depletion hypothesis. Neurosci Biobehav Rev, 9(3), 469-477.

Dackis, C. A., & O'Brien, C. P. (2001). Cocaine dependence: a disease of the brain's reward centers. J Subst Abuse Treat, 21(3), 111-117.

Decorte, T. (2001). Quality control by cocaine users: underdeveloped harm reduction strategies. European Addiction Research, 7(4), 161-175.

DeMaria, P. A., Jr., Sterling, R., & Weinstein, S. P. (2000). The effect of stimulant and sedative use on treatment outcome of patients admitted to methadone maintenance treatment. Am J Addict, 9(2), 145-153.

Dias da Silva, D., Silva, E., & Carmo, H. (2013). Cytotoxic effects of amphetamine mixtures in primary hepatocytes are severely aggravated under hyperthermic conditions. Toxicol In Vitro, 27(6), 1670-1678.

Dias da Silva, D., Silva, M. J., Moreira, P., Martins, M. J., Valente, M. J., Carvalho, F., Carmo, H. (2016). In vitro hepatotoxicity of 'Legal X': the combination of 1-benzylpiperazine (BZP) and 1-(m-trifluoromethylphenyl)piperazine (TFMPP) triggers oxidative stress, mitochondrial impairment and apoptosis. Arch Toxicol.

Diller, A. J., Rocha, A., Cardon, A. L., Valles, R., Wellman, P. J., & Nation, J. R. (2007). The effects of concurrent administration of +/-3,4-methylenedioxymethamphetamine and

cocaine on conditioned place preference in the adult male rat. Pharmacol Biochem Behav, 88(2), 165-170.

Downey, K. K., Helmus, T. C., & Schuster, C. R. (2000). Treatment of heroin-dependent poly-drug abusers with contingency management and buprenorphine maintenance. Exp Clin Psychopharmacol, 8(2), 176-184.

Drescher, K., & Boedeker, W. (1995). Assessment of the Combined Effects of Substances: The Relationship between Concentration Addition and Independent Action. Biometrics, 51(2), 716-730.

Duvauchelle, C. L., Sapoznik, T., & Kornetsky, C. (1998). The synergistic effects of combining cocaine and heroin ("speedball") using a progressive-ratio schedule of drug reinforcement. Pharmacol Biochem Behav, 61(3), 297-302.

EMCDDA. (2002). 2002 Annual report on the state of the drugs problem in the European Union and Norway Polydrug use.

EMCDDA. (2007). Drugs in Focus, Cocaine use in Europe: implications for service delivery

EMCDDA. (2009). Selected issue, Polydrug use: patterns and responses.

EMCDDA. (2012). EMCDDA publishes 2012 report on the state of the drugs problem in Europe, Eurosurveillance.

EMCDDA. (2015). European drug report - Trends and Development.

Erlich, P. (1890). Studien in der cocanreihe. Deutsch Med. Wochenschr, 16, 717.

Fan, L., Sawbridge, D., George, V., Teng, L., Bailey, A., Kitchen, I., & Li, J. M. (2009). Chronic cocaine-induced cardiac oxidative stress and mitogen-activated protein kinase activation: the role of Nox2 oxidase. J Pharmacol Exp Ther, 328(1), 99-106.

Farre, M., de la Torre, R., Gonzalez, M. L., Teran, M. T., Roset, P. N., Menoyo, E., & Cami, J. (1997). Cocaine and alcohol interactions in humans: neuroendocrine effects and cocaethylene metabolism. J Pharmacol Exp Ther, 283(1), 164-176.

Farre, M., de la Torre, R., Llorente, M., Lamas, X., Ugena, B., Segura, J., & Cami, J. (1993). Alcohol and cocaine interactions in humans. J Pharmacol Exp Ther, 266(3), 1364-1373.

Festa, E. D., Russo, S. J., Gazi, F. M., Niyomchai, T., Kemen, L. M., Lin, S. N., . . . Quinones-Jenab, V. (2004). Sex differences in cocaine-induced behavioral responses, pharmacokinetics, and monoamine levels. Neuropharmacology, 46(5), 672-687.

Fineschi, V., Baroldi, G., Centini, F., Cerretani, D., Fiaschi, A. I., Micheli, L., . . . Giorgi, G. (2001). Markers of cardiac oxidative stress and altered morphology after intraperitoneal cocaine injection in a rat model. Int J Legal Med, 114(6), 323-330.

Freye, E., & Levy, J. V. (2009). Pharmacology and abuse of cocaine, amphetamines, ecstasy and related designer drugs : a comprehensive review on their mode of action, treatment of abuse and intoxication. Dordrecht: Springer.

Garfia, A., Valverde, J. L., Borondo, J. C., Candenas, I., & Lucena, J. (1990). Vascular lesions in intestinal ischemia induced by cocaine-alcohol abuse: report of a fatal case due to overdose. J Forensic Sci, 35(3), 740-745.

Garrido, J. M., Marques, M. P., Silva, A. M., Macedo, T. R., Oliveira-Brett, A. M., & Borges, F. (2007). Spectroscopic and electrochemical studies of cocaine-opioid interactions. Anal Bioanal Chem, 388(8), 1799-1808.

Gatch, M. B., Negus, S. S., Butelman, E. R., & Mello, N. K. (1995). Antinociceptive effects of cocaine/opioid combinations in rhesus monkeys. J Pharmacol Exp Ther, 275(3), 1346-1354.

Gerets, H. H., Tilmant, K., Gerin, B., Chanteux, H., Depelchin, B. O., Dhalluin, S., & Atienzar, F. A. (2012). Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell Biol Toxicol, 28(2), 69-87.

Geromel, V., Kadhom, N., Cebalos-Picot, I., Ouari, O., Polidori, A., Munnich, A., Rustin, P. (2001). Superoxide-induced massive apoptosis in cultured skin fibroblasts harboring the neurogenic ataxia retinitis pigmentosa (NARP) mutation in the ATPase-6 gene of the mitochondrial DNA. Hum Mol Genet, 10(11), 1221-1228.

Glauser, J., & Queen, J. R. (2007). An overview of non-cardiac cocaine toxicity. J Emerg Med, 32(2), 181-186.

GM, C. (2000). The Mechanism of Oxidative Phosphorylation The Cell: A Molecular Approach (2nd ed.). Sunderland (MA): Sinauer Associates;.

Goldstein, R. A., DesLauriers, C., & Burda, A. M. (2009). Cocaine: history, social implications, and toxicity--a review. Dis Mon, 55(1), 6-38.

Goni-Allo, B., Puerta, E., Ramos, M., Lasheras, B., Jordan, J., & Aguirre, N. (2008). Minoxidil prevents 3,4-methylenedioxymethamphetamine-induced serotonin depletions: role of mitochondrial ATP-sensitive potassium channels, Akt and ERK. J Neurochem, 104(4), 914-925.

Gossop, M., Griffiths, P., Powis, B., & Strang, J. (1994). Cocaine: patterns of use, route of administration, and severity of dependence. Br J Psychiatry, 164(5), 660-664.

Gossop, M., Manning, V., & Ridge, G. (2006). Concurrent use and order of use of cocaine and alcohol: behavioural differences between users of crack cocaine and cocaine powder. Addiction, 101(9), 1292-1298.

Grant, B. F., & Harford, T. C. (1990). Concurrent and simultaneous use of alcohol with cocaine: results of national survey. Drug Alcohol Depend, 25(1), 97-104.

Graziani, M., Antonilli, L., Togna, A. R., Grassi, M. C., Badiani, A., & Saso, L. (2016). Cardiovascular and Hepatic Toxicity of Cocaine: Potential Beneficial Effects of Modulators of Oxidative Stress. Oxidative Medicine and Cellular Longevity, 2016, 8408479.

Griffith, O. W. (1999). Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radic Biol Med, 27(9-10), 922-935.

Guan, Z., Lui, C. Y., Morkin, E., & Bahl, J. J. (2004). Oxidative stress and apoptosis in cardiomyocyte induced by high-dose alcohol. J Cardiovasc Pharmacol, 44(6), 696-702.

Guo, R., & Ren, J. (2010). Alcohol dehydrogenase accentuates ethanol-induced myocardial dysfunction and mitochondrial damage in mice: role of mitochondrial death pathway. PLoS One, 5(1), e8757.

Harris, D. S., Everhart, E. T., Mendelson, J., & Jones, R. T. (2003). The pharmacology of cocaethylene in humans following cocaine and ethanol administration. Drug Alcohol Depend, 72(2), 169-182.

Hatsukami, D. K., & Fischman, M. W. (1996). Crack cocaine and cocaine hydrochloride. Are the differences myth or reality? Jama, 276(19), 1580-1588.

He, Y. J., Brockmoller, J., Schmidt, H., Roots, I., & Kirchheiner, J. (2008). CYP2D6 ultrarapid metabolism and morphine/codeine ratios in blood: was it codeine or heroin? J Anal Toxicol, 32(2), 178-182.

Heard, K., Palmer, R., & Zahniser, N. R. (2008). Mechanisms of acute cocaine toxicity. Open Pharmacol J, 2(9), 70-78.

Helzer, J. E., & Pryzbeck, T. R. (1988). The co-occurrence of alcoholism with other psychiatric disorders in the general population and its impact on treatment. J Stud Alcohol, 49(3), 219-224.

Hescheler, J., Meyer, R., Plant, S., Krautwurst, D., Rosenthal, W., & Schultz, G. (1991). Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circ Res, 69(6), 1476-1486.

Hiramatsu, M., Kumagai, Y., Unger, S. E., & Cho, A. K. (1990). Metabolism of methylenedioxymethamphetamine: formation of dihydroxymethamphetamine and a quinone identified as its glutathione adduct. J Pharmacol Exp Ther, 254(2), 521-527.

Hollander, J. E. (2008). Cocaine intoxication and hypertension. Ann Emerg Med, 51(3 Suppl), S18-20.

Hudgins, R., McCusker, J., & Stoddard, A. (1995). Cocaine use and risky injection and sexual behaviors. Drug Alcohol Depend, 37(1), 7-14.

Hunt, D. E., Lipton, D. S., Goldsmith, D., & Strug, D. (1984). Street pharmacology: uses of cocaine and heroin in the treatment of addiction. Drug Alcohol Depend, 13(4), 375-387.

Itzhak, Y., & Ali, S. F. (2006). Role of nitrergic system in behavioral and neurotoxic effects of amphetamine analogs. Pharmacol Ther, 109(1-2), 246-262.

Jeffcoat, A. R., Perez-Reyes, M., Hill, J. M., Sadler, B. M., & Cook, C. E. (1989). Cocaine disposition in humans after intravenous injection, nasal insufflation (snorting), or smoking. Drug Metab Dispos, 17(2), 153-159.

Jenkins, A. J., Levine, B., Titus, J., & Smialek, J. E. (1999). The interpretation of cocaine and benzoylecgonine concentrations in postmortem cases. Forensic Sci Int, 101(1), 17-25.

Jenkins, A. J., Oyler, J. M., & Cone, E. J. (1995). Comparison of heroin and cocaine concentrations in saliva with concentrations in blood and plasma. J Anal Toxicol, 19(6), 359-374.

Jover, R., Ponsoda, X., Gomez-Lechon, J., & Castell, J. V. (1993). Cocaine hepatotoxicity: two different toxicity mechanisms for phenobarbital-induced and non-induced rat hepatocytes. Biochem Pharmacol, 46(11), 1967-1974.

Joya, X., Friguls, B., Simo, M., Civit, E., de la Torre, R., Palomeque, A., Garcia-Algar, O. (2011). Acute heroin intoxication in a baby chronically exposed to cocaine and heroin: a case report. J Med Case Rep, 5, 288. doi: 10.1186/1752-1947-5-288

Jufer, R. A., Wstadik, A., Walsh, S. L., Levine, B. S., & Cone, E. J. (2000). Elimination of cocaine and metabolites in plasma, saliva, and urine following repeated oral administration to human volunteers. J Anal Toxicol, 24(7), 467-477.

Kamendulis, L. M., Brzezinski, M. R., Pindel, E. V., Bosron, W. F., & Dean, R. A. (1996). Metabolism of cocaine and heroin is catalyzed by the same human liver carboxylesterases. J Pharmacol Exp Ther, 279(2), 713-717.

Kampman, K. M., Pettinati, H. M., Lynch, K. G., Spratt, K., Wierzbicki, M. R., & O'Brien, C. P. (2013). A double-blind, placebo-controlled trial of topiramate for the treatment of comorbid cocaine and alcohol dependence. Drug Alcohol Depend, 133(1), 94-99.

Kanel, G. C., Cassidy, W., Shuster, L., & Reynolds, T. B. (1990). Cocaine-induced liver cell injury: comparison of morphological features in man and in experimental models. Hepatology, 11(4), 646-651.

Kannan, M., Wang, L., & Kang, Y. J. (2004). Myocardial oxidative stress and toxicity induced by acute ethanol exposure in mice. Exp Biol Med (Maywood), 229(6), 553-559.

Karch, S. B. (1999). Cocaine: history, use, abuse. J R Soc Med, 92(8), 393-397.

Kelly, B. C., & Parsons, J. T. (2008). Predictors and comparisons of polydrug and non-polydrug cocaine use in club subcultures. Am J Drug Alcohol Abuse, 34(6), 774-781.

Khorana, N., Pullagurla, M. R., Young, R., & Glennon, R. A. (2004). Comparison of the discriminative stimulus effects of 3,4-methylenedioxymethamphetamine (MDMA) and cocaine: asymmetric generalization. Drug Alcohol Depend, 74(3), 281-287.

Kimes, B. W., & Brandt, B. L. (1976). Properties of a clonal muscle cell line from rat heart. Exp Cell Res, 98(2), 367-381.

King, V. L., Kidorf, M. S., Stoller, K. B., Carter, J. A., & Brooner, R. K. (2001). Influence of antisocial personality subtypes on drug abuse treatment response. J Nerv Ment Dis, 189(9), 593-601.

Kintz, P., Sengler, C., Cirimele, V., & Mangin, P. (1997). Evidence of crack use by anhydroecgonine methylester identification. Hum Exp Toxicol, 16(2), 123-127.

Kloner, R. A., Hale, S., Alker, K., & Rezkalla, S. (1992). The effects of acute and chronic cocaine use on the heart. Circulation, 85(2), 407-419.

Kobayashi, T., Nishizawa, D., & Ikeda, K. (2011). Inhibition of g protein-activated inwardly rectifying k channels by phencyclidine. Curr Neuropharmacol, 9(1), 244-246.

Koczor, C. A., Ludlow, I., Hight, R. S., 2nd, Jiao, Z., Fields, E., Ludaway, T., Lewis, W. (2015). Ecstasy (MDMA) Alters Cardiac Gene Expression and DNA Methylation: Implications for Circadian Rhythm Dysfunction in the Heart. Toxicol Sci, 148(1), 183-191.

Koop, D. R., & Tierney, D. J. (1990). Multiple mechanisms in the regulation of ethanol-inducible cytochrome P450IIE1. Bioessays, 12(9), 429-435.

Kosten, T. R., Kosten, T. A., McDougle, C. J., Hameedi, F. A., McCance, E. F., Rosen, M. I., Price, L. H. (1996). Gender differences in response to intranasal cocaine administration to humans. Biol Psychiatry, 39(2), 147-148.

LeDuc, B. W., Sinclair, P. R., Shuster, L., Sinclair, J. F., Evans, J. E., & Greenblatt, D. J. (1993). Norcocaine and N-hydroxynorcocaine formation in human liver microsomes: role of cytochrome P-450 3A4. Pharmacology, 46(5), 294-300.

LeDuc, B. W., Sinclair, P. R., Walton, H. S., Sinclair, J. F., Greenblatt, D. J., & Shuster, L. (1994). Cocaine toxicity in cultured chicken hepatocytes: role of cytochrome P450. Toxicol Appl Pharmacol, 125(2), 322-332.

Leri, F., Bruneau, J., & Stewart, J. (2003). Understanding polydrug use: review of heroin and cocaine co-use. Addiction, 98(1), 7-22.

Li, A. P., Kaminski, D. L., & Rasmussen, A. (1995). Substrates of human hepatic cytochrome P450 3A4. Toxicology, 104(1-3), 1-8.

Li, S. Y., Li, Q., Shen, J. J., Dong, F., Sigmon, V. K., Liu, Y., & Ren, J. (2006). Attenuation of acetaldehyde-induced cell injury by overexpression of aldehyde dehydrogenase-2 (ALDH2) transgene in human cardiac myocytes: role of MAP kinase signaling. J Mol Cell Cardiol, 40(2), 283-294.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J Biol Chem, 193(1), 265-275.

Lukas, S. E., Sholar, M., Lundahl, L. H., Lamas, X., Kouri, E., Wines, J. D., Mendelson, J. H. (1996). Sex differences in plasma cocaine levels and subjective effects after acute cocaine administration in human volunteers. Psychopharmacology (Berl), 125(4), 346-354.

Maldonado, R. (1997). Participation of noradrenergic pathways in the expression of opiate withdrawal: biochemical and pharmacological evidence. Neurosci Biobehav Rev, 21(1), 91-104.

Malison, R. T., Best, S. E., van Dyck, C. H., McCance, E. F., Wallace, E. A., Laruelle, M., . . . Innis, R. B. (1998). Elevated striatal dopamine transporters during acute cocaine abstinence as measured by [123I] beta-CIT SPECT. Am J Psychiatry, 155(6), 832-834.

Marks, V., & Chapple, P. A. (1967). Hepatic dysfunction in heroin and cocaine users. Br J Addict Alcohol Other Drugs, 62(1), 189-195.

Matthews, J. C., & Collins, A. (1983). Interactions of cocaine and cocaine congeners with sodium channels. Biochem Pharmacol, 32(3), 455-460.

Maurer, H. H., Sauer, C., & Theobald, D. S. (2006). Toxicokinetics of drugs of abuse: current knowledge of the isoenzymes involved in the human metabolism of tetrahydrocannabinol, cocaine, heroin, morphine, and codeine. Ther Drug Monit, 28(3), 447-453.

McCance-Katz, E. F., Kosten, T. R., & Jatlow, P. (1998). Concurrent use of cocaine and alcohol is more potent and potentially more toxic than use of either alone--a multiple-dose study. Biol Psychiatry, 44(4), 250-259.

McCance, E. F., Price, L. H., Kosten, T. R., & Jatlow, P. I. (1995). Cocaethylene: pharmacology, physiology and behavioral effects in humans. J Pharmacol Exp Ther, 274(1), 215-223.

Misra, A. L., Pontani, R. B., & Vadlamani, N. L. (1987). Stereospecific potentiation of opiate analgesia by cocaine: predominant role of noradrenaline. Pain, 28(1), 129-138.

Moolchan, E. T., Cone, E. J., Wstadik, A., Huestis, M. A., & Preston, K. L. (2000). Cocaine and metabolite elimination patterns in chronic cocaine users during cessation: plasma and saliva analysis. J Anal Toxicol, 24(7), 458-466.

Morgan, A. E., Horan, B., Dewey, S. L., & Ashby, C. R., Jr. (1997). Repeated administration of 3,4-methylenedioxymethamphetamine augments cocaine's action on dopamine in the nucleus accumbens: a microdialysis study. Eur J Pharmacol, 331(1), R1-3.

Muller, C. P., Carey, R. J., Huston, J. P., & De Souza Silva, M. A. (2007). Serotonin and psychostimulant addiction: focus on 5-HT1A-receptors. Prog Neurobiol, 81(3), 133-178.

Myers, A. L., Williams, H. E., Kraner, J. C., & Callery, P. S. (2005). Identification of anhydroecgonine ethyl ester in the urine of a drug overdose victim. J Forensic Sci, 50(6), 1481-1485.

Ndikum-Moffor, F. M., Schoeb, T. R., & Roberts, S. M. (1998). Liver toxicity from norcocaine nitroxide, an N-oxidative metabolite of cocaine. J Pharmacol Exp Ther, 284(1), 413-419.

Neri, M., Cerretani, D., Fiaschi, A. I., Laghi, P. F., Lazzerini, P. E., Maffione, A. B., Fineschi, V. (2007). Correlation between cardiac oxidative stress and myocardial pathology due to acute and chronic norepinephrine administration in rats. J Cell Mol Med, 11(1), 156-170.

Nicolas, J. M., Rubin, E., & Thomas, A. P. (1996). Ethanol and cocaine cause additive inhibitory effects on the calcium transients and contraction in single cardiomyocytes. Alcohol Clin Exp Res, 20(6), 1077-1082.

Niemela, O. (2007). Acetaldehyde adducts in circulation. Novartis Found Symp, 285, 183-192; discussion 193-187.

Nott, M. W. (1968). Potentiation of morphine analgesia by cocaine in mice. Eur J Pharmacol, 5(1), 93-99.

O'Leary, M. E., & Hancox, J. C. (2010). Role of voltage-gated sodium, potassium and calcium channels in the development of cocaine-associated cardiac arrhythmias. Br J Clin Pharmacol, 69(5), 427-442.

Olszewski, D., Matias, J., Monshouwer, K., & Kokkevi, A. (2009). Polydrug use among 15- to 16-year-olds: Similarities and differences in Europe. Drugs: Education, Prevention and Policy.

Pakula, B., Macdonald, S., & Stockwell, T. (2009). Settings and functions related to simultaneous use of alcohol with marijuana or cocaine among clients in treatment for substance abuse. Subst Use Misuse, 44(2), 212-226.

Pakula, B., Macdonald, S., Stockwell, T., & Sharma, R. (2009). Simulatneous use of alcohol and cocaine: A qualitative investigation. Journal of Substance USe, 14(2), 101-112.

Panos, J. J., & Baker, L. E. (2010). An in vivo microdialysis assessment of concurrent MDMA and cocaine administration in Sprague-Dawley rats. Psychopharmacology (Berl), 209(1), 95-102.

Pattison, L. P., McIntosh, S., Budygin, E. A., & Hemby, S. E. (2012). Differential regulation of accumbal dopamine transmission in rats following cocaine, heroin and speedball self-administration. J Neurochem, 122(1), 138-146.

Pattison, L. P., McIntosh, S., Sexton, T., Childers, S. R., & Hemby, S. E. (2014). Changes in dopamine transporter binding in nucleus accumbens following chronic self-administration cocaine: heroin combinations. Synapse, 68(10), 437-444.

Pellinen, P., Honkakoski, P., Stenback, F., Niemitz, M., Alhava, E., Pelkonen, O., Pasanen, M. (1994). Cocaine N-demethylation and the metabolism-related hepatotoxicity can be prevented by cytochrome P450 3A inhibitors. Eur J Pharmacol, 270(1), 35-43.

Pellinen, P., Kulmala, L., Konttila, J., Auriola, S., Pasanen, M., & Juvonen, R. (2000). Kinetic characteristics of norcocaine N-hydroxylation in mouse and human liver microsomes: involvement of CYP enzymes. Arch Toxicol, 74(9), 511-520.

Pellinen, P., Stenback, F., Kojo, A., Honkakoski, P., Gelboin, H. V., & Pasanen, M. (1996). Regenerative changes in hepatic morphology and enhanced expression of CYP2B10 and CYP3A during daily administration of cocaine. Hepatology, 23(3), 515-523.

Perez-Reyes, M. (1993). Subjective and cardiovascular effects of cocaethylene in humans. Psychopharmacology (Berl), 113(1), 144-147.

Perez-Reyes, M., & Jeffcoat, A. R. (1992). Ethanol/cocaine interaction: cocaine and cocaethylene plasma concentrations and their relationship to subjective and cardiovascular effects. Life Sci, 51(8), 553-563.

Perez-Reyes, M., Jeffcoat, A. R., Myers, M., Sihler, K., & Cook, C. E. (1994). Comparison in humans of the potency and pharmacokinetics of intravenously injected cocaethylene and cocaine. Psychopharmacology (Berl), 116(4), 428-432.

Perino, L. E., Warren, G. H., & Levine, J. S. (1987). Cocaine-induced hepatotoxicity in humans. Gastroenterology, 93(1), 176-180.

Pilgrim, J. L., Woodford, N., & Drummer, O. H. (2013). Cocaine in sudden and unexpected death: a review of 49 post-mortem cases. Forensic Sci Int, 227(1-3), 52-59.

Poet, T. S., Brendel, K., & Halpert, J. R. (1994). Inactivation of cytochromes P450 2B protects against cocaine-mediated toxicity in rat liver slices. Toxicol Appl Pharmacol, 126(1), 26-32.

Poet, T. S., McQueen, C. A., & Halpert, J. R. (1996). Participation of cytochromes P4502B and P4503A in cocaine toxicity in rat hepatocytes. Drug Metab Dispos, 24(1), 74-80.

Politi, L., Zucchella, A., Morini, L., Stramesi, C., & Polettini, A. (2007). Markers of chronic alcohol use in hair: comparison of ethyl glucuronide and cocaethylene in cocaine users. Forensic Sci Int, 172(1), 23-27.

Pontes, H., de Pinho, P. G., Fernandes, E., Branco, P. S., Ferreira, L. M., Carmo, H., Bastos, M. L. (2010). Metabolic interactions between ethanol and MDMA in primary cultured rat hepatocytes. Toxicology, 270(2-3), 150-157.

Restrepo, C. S., Rojas, C. A., Martinez, S., Riascos, R., Marmol-Velez, A., Carrillo, J., & Vargas, D. (2009). Cardiovascular complications of cocaine: imaging findings. Emerg Radiol, 16(1), 11-19.

Reyes, S., Kane, G. C., Zingman, L. V., Yamada, S., & Terzic, A. (2009). Targeted disruption of K(ATP) channels aggravates cardiac toxicity in cocaine abuse. Clin Transl Sci, 2(5), 361-365.

Rhodes, T., Briggs, D., Kimber, J., Jones, S., & Holloway, G. (2007). Crack-heroin speedball injection and its implications for vein care: qualitative study. Addiction, 102(11), 1782-1790.

Rothman, R. B., & Baumann, M. H. (2003). Monoamine transporters and psychostimulant drugs. Eur J Pharmacol, 479(1-3), 23-40.

Sardao, V. A., Oliveira, P. J., Holy, J., Oliveira, C. R., & Wallace, K. B. (2007). Vital imaging of H9c2 myoblasts exposed to tert-butylhydroperoxide--characterization of morphological features of cell death. BMC Cell Biol, 8, 11.

Satran, A., Bart, B. A., Henry, C. R., Murad, M. B., Talukdar, S., Satran, D., & Henry, T. D. (2005). Increased prevalence of coronary artery aneurysms among cocaine users. Circulation, 111(19), 2424-2429.

Schilstrom, B., Yaka, R., Argilli, E., Suvarna, N., Schumann, J., Chen, B. T., . . . Bonci, A. (2006). Cocaine enhances NMDA receptor-mediated currents in ventral tegmental area cells via dopamine D5 receptor-dependent redistribution of NMDA receptors. J Neurosci, 26(33), 8549-8558.

Schmid, Y., Vizeli, P., Hysek, C. M., Prestin, K., Meyer Zu Schwabedissen, H. E., & Liechti, M. E. (2016). CYP2D6 function moderates the pharmacokinetics and pharmacodynamics of 3,4-methylene-dioxymethamphetamine in a controlled study in healthy individuals. Pharmacogenet Genomics, 26(8), 397-401.

Shen, H., He, M. M., Liu, H., Wrighton, S. A., Wang, L., Guo, B., & Li, C. (2007). Comparative metabolic capabilities and inhibitory profiles of CYP2D6.1, CYP2D6.10, and CYP2D6.17. Drug Metab Dispos, 35(8), 1292-1300.

Shenouda, S. K., Lord, K. C., McIlwain, E., Lucchesi, P. A., & Varner, K. J. (2008). Ecstasy produces left ventricular dysfunction and oxidative stress in rats. Cardiovasc Res, 79(4), 662-670.

Shimada, A., Tsuda, T., & Yanagita, T. (1988). Mode of potentiating action of cocaine in morphine analgesia. Jpn J Pharmacol, 48(2), 185-193.

Sierra, V., Duttaroy, A., Lutfy, K., Candido, J., Billings, B., Zito, S. W., & Yoburn, B. C. (1992). Potentiation of opioid analgesia by cocaine: the role of spinal and supraspinal receptors. Life Sci, 50(8), 591-597.

Smith, A. C., Freeman, R. W., & Harbison, R. D. (1981). Ethanol enhancement of cocaine-induced hepatotoxicity. Biochem Pharmacol, 30(5), 453-458.

Sobel, B. F., & Riley, A. L. (1999). The interaction of cocaethylene and cocaine and of cocaethylene and alcohol on schedule-controlled responding in rats. Psychopharmacology (Berl), 145(2), 153-161.

Song, B.-J., Moon, K.-H., Upreti, V. V., Eddington, N. D., & Lee, I. J. (2010). Mechanisms of MDMA (Ecstasy)-Induced Oxidative Stress, Mitochondrial Dysfunction, and Organ Damage. Current pharmaceutical biotechnology, 11(5), 434-443.

Sora, I., Hall, F. S., Andrews, A. M., Itokawa, M., Li, X. F., Wei, H. B., Uhl, G. R. (2001). Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. Proc Natl Acad Sci U S A, 98(9), 5300-5305.

Starosciak, A. K., Zakharova, E., Stagg, M., Matos, J., & Izenwasser, S. (2012). Differential alteration of the effects of MDMA (ecstasy) on locomotor activity and cocaine conditioned place preference in male adolescent rats by social and environmental enrichment. Psychopharmacology (Berl), 224(1), 101-108.

Sultana, R., Bhupanapadu Sunkesula, S. R., Sharma, V., Reddanna, P., & Babu, P. P. (2005). Formation of acetaldehyde adducts of glutathione S-transferase A3 in the liver of rats administered alcohol chronically. Alcohol, 35(1), 57-66.

Tashkin, D. P., Kleerup, E. C., Koyal, S. N., Marques, J. A., & Goldman, M. D. (1996). Acute effects of inhaled and i.v. cocaine on airway dynamics. Chest, 110(4), 904-910.

Thompson, M. L., Shuster, L., & Shaw, K. (1979). Cocaine-induced hepatic necrosis in mice--the role of cocaine metabolism. Biochem Pharmacol, 28(15), 2389-2395.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. J Physiol, 552(Pt 2), 335-344.

Uszenski, R. T., Gillis, R. A., Schaer, G. L., Analouei, A. R., & Kuhn, F. E. (1992). Additive myocardial depressant effects of cocaine and ethanol. Am Heart J, 124(5), 1276-1283.

Valente, M. J., Araujo, A. M., Bastos Mde, L., Fernandes, E., Carvalho, F., Guedes de Pinho, P., & Carvalho, M. (2016). Editor's Highlight: Characterization of Hepatotoxicity Mechanisms Triggered by Designer Cathinone Drugs (beta-Keto Amphetamines). Toxicol Sci, 153(1), 89-102.

Valente, M. J., Carvalho, F., Bastos, M., de Pinho, P. G., & Carvalho, M. (2012). Contribution of oxidative metabolism to cocaine-induced liver and kidney damage. Curr Med Chem, 19(33), 5601-5606.

Van der Poel, A., Rodenburg, G., Dijkstra, M., Stoele, M., & Van de Mheen, D. (2009). Trends, motivations and settings of recreational cocaine use by adolescents and young adults in the Netherlands. Int J Drug Policy, 20(2), 143-151.

Vendemiale, G., Grattagliano, I., Altomare, E., Serviddio, G., Portincasa, P., Prigigallo, F., & Palasciano, G. (2001). Mitochondrial oxidative damage and myocardial fibrosis in rats chronically intoxicated with moderate doses of ethanol. Toxicol Lett, 123(2-3), 209-216.

Volkow, N. D. (2009). Cocaine abuse and addiction. NIH publication. Retrieved 25 January, 2015, from http://www.drugabuse.gov/publications/research-reports/cocaine/what-cocaine

Volkow, N. D., Fowler, J. S., & Wang, G. J. (2003). The addicted human brain: insights from imaging studies. J Clin Invest, 111(10), 1444-1451.

Volkow, N. D., Fowler, J. S., Wang, G. J., & Swanson, J. M. (2004). Dopamine in drug abuse and addiction: results from imaging studies and treatment implications. Mol Psychiatry, 9(6), 557-569.

Ward, S. J., Morgan, D., & Roberts, D. C. (2005). Comparison of the reinforcing effects of cocaine and cocaine/heroin combinations under progressive ratio and choice schedules in rats. Neuropsychopharmacology, 30(2), 286-295.

Welder, A. A., Smith, M. A., Ramos, K., & Acosta, D. (1988). Cocaine-induced cardiotoxicity in vitro. Toxicol In Vitro, 2(3), 205-213.

Williams, H., Dratcu, L., Taylor, R., Roberts, M., & Oyefeso, A. (1998). "Saturday night fever": ecstasy related problems in a London accident and emergency department. J Accid Emerg Med, 15(5), 322-326.

Winstock, A. R., Griffiths, P., & Stewart, D. (2001). Drugs and the dance music scene: a survey of current drug use patterns among a sample of dance music enthusiasts in the UK. Drug Alcohol Depend, 64(1), 9-17.

Wojtczak, L., Teplova, V. V., Bogucka, K., Czyz, A., Makowska, A., Wieckowski, M. R., . . . Evtodienko, Y. V. (1999). Effect of glucose and deoxyglucose on the redistribution of calcium in ehrlich ascites tumour and Zajdela hepatoma cells and its consequences for mitochondrial energetics. Further arguments for the role of Ca(2+) in the mechanism of the crabtree effect. Eur J Biochem, 263(2), 495-501.

Xu, W., Flick, T., Mitchel, J., Knowles, C., & Ault, K. (1999). Cocaine effects on immunocompetent cells: an observation of in vitro cocaine exposure. Int J Immunopharmacol, 21(7), 463-472.

Yao, D., Shi, X., Wang, L., Gosnell, B. A., & Chen, C. (2013). Characterization of differential cocaine metabolism in mouse and rat through metabolomics-guided metabolite profiling. Drug Metab Dispos, 41(1), 79-88.

Yuan, C., & Acosta, D., Jr. (2000). Effect of cocaine on mitochondrial electron transport chain evaluated in primary cultures of neonatal rat myocardial cells and in isolated mitochondrial preparations. Drug Chem Toxicol, 23(2), 339-348.

Zaragoza, A., Diez-Fernandez, C., Alvarez, A. M., Andres, D., & Cascales, M. (2001). Mitochondrial involvement in cocaine-treated rat hepatocytes: effect of N-acetylcysteine and deferoxamine. Br J Pharmacol, 132(5), 1063-1070.

Zhan, M., Hou, S., Zhan, C. G., & Zheng, F. (2014). Kinetic characterization of high-activity mutants of human butyrylcholinesterase for the cocaine metabolite norcocaine. Biochem J, 457(1), 197-206.